Studies on Catalase in Chloroplasts

Hisateru MITSUDA and Katsuharu YASUMATSU*

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It is said that many enzymes associate with cell components.

In green leaves catalase, also, exists in chloroplasts and it is not eluated by distilled water or buffer from them.

As association with particulate material is an unmitgated nuisance for isolation of highly purified enzymes, we tested various methods to make catalase into solution, for example, dioxane, picoline, ethanol, n-butanol treatment and dry ice treatment.

In this paper, we tested them on the effects upon the catalase activity and extractabilities.

Accordingly, we found that n-butanol treatment was ideally suited to extract catalase from chloroplasts on the following three points.

Firstly, the catalase activity is not inactivated. Secondly, the method has an excellent extractability. Thirdly, the method is specific.

INTRODUCTION

It is said[1] that the plant catalase exists in the chloroplasts of the green leaves. But it is open to discussion whether the plant catalase exists in the grana of the chloroplasts.[2]

Recent studies on the distribution of intracellular enzymes have clearly demonstrated that many are closely associated with well-defined cell components.

Many methods to make the enzymes associated with the insoluble particles into the solution, have been devised. For example, we have the autolysis, the digestion with lipase, trypsin and papain, and the method of utilizing the detergent as well as the destruction by the sonic vibration.

But we see these methods act more or less unfavourably on the purification of enzymes, and as the plant catalase is particularly unstable, it is not desirable for us to apply these methods of the treatment to the purification.

We have continued to study on the plant catalase since 1939, and minutely examined and confirmed the facts that this catalase exists in the chloroplasts and its catalase activity runs parallel with the quantity of the chlorophyll and reported them.[3]

Again, we confirmed that the plant catalase existed in the grana of the chloroplasts associated with the cell components, and tried every means to make this catalase associated with the insoluble particles into solution keeping it as active as

* 満田 久輝, 安松 克治

possible.

Recently, Morton[4] reported in his studies on milk phosphatase[5][6] and peptidase[7] of hog kidney how to make the enzyme associated with insoluble cell components into the solution by means of the butanol.

Applying this butanol method to the plant catalase, we found that we could extract the catalase from the chloroplasts most efficiently.

We report the gist of our study in the following, hoping to receive comments and criticisms of the readers.

EXPERIMENTAL RESULTS

In order to separate the chloroplast as undamaged as possible, we must grind the sample down in such isotonic solution as 0.5 M sucrose or glucose.

The damages of the chloroplasts naturally vary in the sorts of the plants.

While the spinach is easily damageable, the green leaves of the rice-plant, Tabacco and Tea are the stable samples and it is comparatively easy and sure for us to separate the chloroplasts of them.

Accordingly, in order to study the distribution of the catalase in the cell, we should make careful considerations on the above-mentioned points.

Though we can let the chloroplasts cohere, and separate them by $CaCl_2$ or by the lead acetate, we think it is the most representative method to separate them by adding the acetic acid to them and adjusting them at pH 5.2.

For example, grind down the leaf of the rice-plant in 0.5 M sucrose (or glucose) solution and extract the green suspension from it, and then add 0.2 M acetic acid to the green suspension to keep the solution at pH 5.2 for a little while, and you see the chloroplasts are separated.

We kept the solution in an ice-box for one night, and separated it by centrifugation into chloroplasts and cytoplasmic solution. Then we measured the catalase activity in each fraction and found no sign of the activity in the cytoplasmic solution, but all the catalase was recognized in the chloroplast.

Though we washed the chloroplasts comprising the catalase by distilled water repeatedly, it was not eluated, and we even washed them by 0.01 M phosphate buffer but it was not eluated at all.

Thus we recognized that almost all the catalase existed in the chloroplasts.

Having made the similar experiments on spinach, we recognized the same result that almost all the catalase was in the chloroplasts and only a little of it in the cytoplasmic solution.

Then it became our question how to extract the catalase in the chloroplasts.

Generally speaking, when we choose a method of extracting a catalase from a chloroplast we must make considerations on the following three points. Firstly, the catalase activity should not be inactivated. Secondly, the method should have an

excellent extractability. Thirdly, the method should be specific.

Organic solvents have not been applied so generally for the separation of enzymes, since they denature proteins at room temperature.

But recently, they have been put to use at low temperatures and low ionic strengths for the protein fractionation[8] or the purification of enzymes.[9]

However, as the plant catalase is very unstable, those organic solvents are in danger of inactivating it.

Therefore, we tested the effects of these solvents upon the catalase activity before we had used them for the extraction.

Fig. 1. Scheme to prepare the sample used for test on effects of organic solvents.

Leaf-blade of Spinach 100 g
- Add 150 cc. of 0.01 M phosphate buffer (pH 7.0) to it and grind it down by a homogenizer.
- Stir it for 1 hour at the ordinary temperature.
- Keep it overnight in an ice box.
- Filter it through cloth.

Green suspension
- Cool it to 0°C with ice.
- Add the cold organic solvent slowly to it.

Of course, in case of the test on the stability of catalase activity, we should note that the concentration of the catalase is an important factor to act upon the stability.

Accordingly, we standardised the concentration of the catalase and that of the phosphate buffer in any case of our tests.

We showed in Table 1 the result of our test of the effects of organic solvents upon plant catalase by our treatment mentioned in Fig. 1.

As we show in Table 1, picoline inactivates catalase, but catalase is stable against dioxane and ethanol, and the stability of catalase is very high against butanol as in case of the water saturated with butanol.

Based on the result of the tests upon the stability above-mentioned, we further made experiments on the extraction according to the scheme shown in Fig. 2.

According to the scheme shown in Fig. 2, add each organic solvent to the green suspension of the chloroplasts and prepare the supernatant liquid by centrifuging it at 3500 r.p.m., for 20 mins. and then dialyse it against 2 L of 0.01 M phosphate buffer for 48 hrs., and measuring a certain quantity of dry matter in the solution, and calculate the catalase activity (Kat.-f.).

Thus you see the results of our experiment above-mentioned in Table 2.
From the above-mentioned results, you see that we place, in order of extractability, butanol treatment, ethanol, dioxane and dry ice treatment, but in order of the

Table 1. Effects of organic solvents on stability of catalase.

Lapse of time		0		2		20	
Solvents	Concentration %	T. A.	Ratio	T. A.	Ratio	T. A.	Ratio
Dioxane	10	32.0	100.0	32.3	101.0	32.0	100.0
	20	32.0	100.0	32.0	100.0	30.7	96.0
	30	31.3	100.0	31.4	100.5	27.5	87.8
	35	31.3	100.0	27.5	97.8	26.0	83.0
	40	32.1	100.0	26.8	83.5	24.0	74.8
	50	32.2	100.0	26.3	81.6	24.0	74.6
Picoline	5	32.5	100.0	32.5	100.0	32.5	100.0
	10	32.5	100.0	30.9	95.0	29.3	90.1
	20	33.0	100.0	32.4	98.2	29.5	89.5
	30	32.5	100.0	31.9	98.2	21.8	67.0
	40	35.5	100.0	34.8	98.0	17.4	49.0
	50	35.2	100.0	28.7	81.6	9.6	27.4
	60	35.5	100.0	27.0	76.1	7.1	20.0
Ethanol	10	41.2	100.0	41.0	99.6	41.7	100.1
	20	41.0	100.0	40.4	98.4	41.2	100.7
	30	41.3	100.0	40.3	97.4	39.9	96.5
	40	41.4	100.0	38.9	94.0	36.4	87.9
	50	41.2	100.0	38.6	92.1	29.1	70.6
n-Butanol	Sat.	35.0	100.0	34.3	98.0	34.0	97.1

T. A. = Total activity = K × (dilution) × (quantity of solution) We determined catalase activities by the iodometry (H. S. Olcott & C. D. W. Thornton : *J. Am. Chem. Soc.*, **61**, 2147 (1939)).

Table 2. Experiments on the extractabilities of various organic solvents acted upon the plant catalase.

Organic solvents	Original solution		Supernatant liquid		Rate of extraction %	Increase in Kat. -f.
	Kat. -f.	T. A.	Kat. -f.	T. A.		
Dioxane	179	72.4	650	52.8	73.0	3.6-fold
Ethanol	175	72.2	262	53.8	74.4	1.5-fold
Dry ice Treatment	179	72.4	494	37.3	51.6	2.8-fold
n-Butanol Treatment	179	72.4	911	57.9	80.0	5.1-fold

increasing degree of Kat.-f., that is to say, the specificity of extraction, we arrange butanol treatment, dioxane, dry ice and ethanol.

Fig. 2a. Scheme to illustrate the extraction of catalase from chloroplasts.

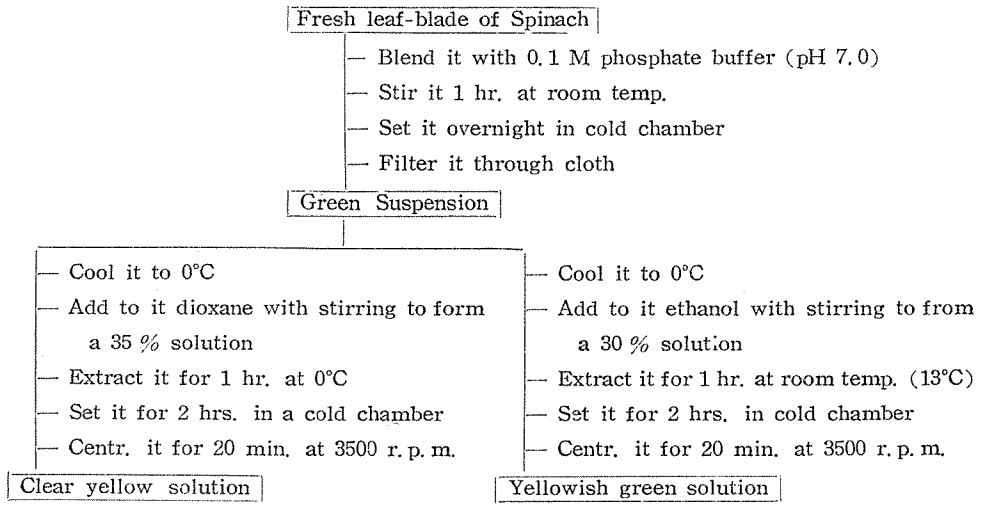
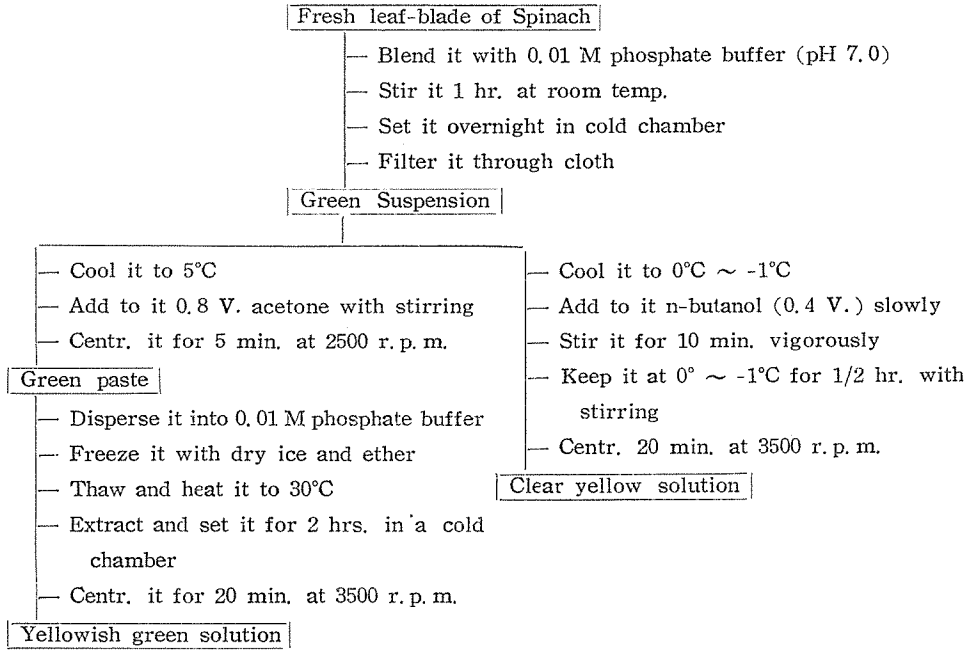


Fig. 2b. Scheme to illustrate the extraction of catalase from chloroplasts.



The conditions to use an organic solvent for extraction of catalase are that the solvent should not inactivate the catalase activity, and its rate of extraction should be very high, and its extraction should be specific.

We prove that butanol treatment eluates catalase in the chloroplasts best as it meets all the conditions above-stated and has such an advantage as it will not increase the extracted matter too much.

DISCUSSION

To extract enzyme from the cell, we often apply in case of bacteria and yeast the method to freeze the cell by dry ice and to thaw it so that the cell membrane is broken and enzyme is extracted.

This time, we applied the above method to the chloroplasts, but the result is not so satisfactory as we expected just as you see in Table 2.

Takashima[10] reports that he once studied chlorophyll lipoprotein by means of adding α-picoline to the grana suspension of the leaf of clover, and when he added 50 to 55 % of picoline to it the turbidity of the solution was cleared, but at the same time, picoline inactivated all the catalase.

Indeed picoline inactivates catalase and accordingly it is not a very adequate solvent, but as we show in Table 2, picoline does not so completely inactivate the catalase as Takashima reports.

It is a matter of temperature. When it is around 0°C, even when two hours after we added 50 % of α-picoline to the solution, we can see 81.6 % of catalase remain actiue.

Even when we make the concentration of picoline increase to 60 %, 76.1 % of catalase remain active, but we think this is not so satisfactory for our use as other solvents.

Dioxane is the solvent which made Sumner[11] succeed in crystallizing the cow liver catalase for the first time in the world.

One of the writers[12] too once succeeded in refining and crystallizing very active plant catalase by means of applying dioxane to the extraction and the fractionation of the catalase.

Dioxane is more stable and capable of extracting catalase from chloroplasts than the popular solvent ethanol.

It has also such a merit that when we make 50 % solution of it, it is able to precipitate all the catalase in the solution.

But under the present situation in Japan, dioxane is a too dear solvent to be used for the purification of enzymes.

The butanol treatment is extremely effective to isolate catalase in the chloroplasts, as we explain in the experimental result.

From 30 % to 40 % of chloroplast is lipoid.

As Morton[4] points out, butanol is capable of removing lipoid from chloroplasts, and it is easily saturated in a solution.

When the solution separates into distinct layers, remove the butanol layer

from the enzyme solution and you get the aqueous solution.

In course of subsequent purification, add 0.8 volume of acetone to the latter or else half saturate the aqueous solution with ammonium sulfate, and in either case, catalase precipitate and you can go on with fractionation.

When A. W. Galston[13] obtained very active plant catalase, he treated chloroplasts with acetone to extract catalase from it and then freeze and dry it, that is to say, he adopted the so-called lyophile method.

Though this is certainly an excellent method, butanol treatment is much more universal and excellent than it from the view-point of the experimental apparatus.

Generally speaking, as we stated above, it is desirable for a method to extract catalase from the cell to be highly extractable and able to make catalase very stable and specific.

Accordingly, butanol treatment is extremely excellent.

SUMMARY

(1) In case of eluating plant catalase from chloroplasts, we adopted various methods and compared each other.

(2) We tested upon such organic solvents as dioxane, picoline, ethanol and butanol which were adopted to extract catalase, in order to study how they affect the stability of catalase.

(3) Picoline inactivates catalase and accordingly it is unfavourable, but in the other solvents, catalase is tolerably stable.

(4) We compared the methods of dioxane, ethanol and butanol and the freezing and thawing method using a dry ice each other, and measured the extractibility and the specificity of catalase treated by each method and again compared and studied our experimental results, and after all we found that the butanol treatment was as good a method as could be.

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