Notes on Extractant-Dependent Leaf Peroxidase and Malate Dehydrogenase Zymograms from Twenty Plant Species

Toru ENDO and Masaaki IHARA

National Institute of Genetics, Misima, 〒411

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Zymograms can be obtained by three procedures, i.e., enzyme extraction, gel electrophoresis and enzyme staining. Although the latter two procedures have been applied to the determination of enzymes in many species the first procedure has not yet been standardized, especially for leaf enzymes. Thus the extraction procedures for leaf enzymes so far reported tend to lack uniformity. For instance, juice obtained without any extractant (HAMILL and BREWBAKER 1969, HVID and NIELSEN 1977, TORRES *et al.* 1978, HICK *et al.* 1982), or complex extractants with pretreatments, followed by several posttreatments (BHATIA *et al.* 1967, McCOWN *et al.* 1969, KELLEY and ADAMS 1977) have been used. Intermediate procedures between these two extreme cases are generally applied. In these experiments, each extractant contains one, several or most of the following substances; buffer, salts, phenolics-absorbable polymers, antioxidants, nonionic or cationic detergents, chelating compounds, sugars, and so on. It should be noted that the buffers vary widely in their composition, pH, and molarity. There is no rule as to the administration of these components, and this situation may or may not reflect the differences in the intracellular forms of leaf enzyme systems of various plant species.

It is likely that different extraction procedures result in changes in isozyme survival. These results, however, have not yet been evaluated in a wide range of plant species. Thus the current investigation was undertaken with a view to analysing the effects of the above-mentioned chemicals on the extraction of leaf enzymes from twenty plant species. Chelating compounds and sugars were not examined, because of their comparatively low effectiveness in enzyme extraction. Also, pretreatments were omitted in order to avoid the loss of enzyme activities.

One Pteropsida, seven Gymnospermae, nine Dicotyledonous and three Monocotyledonous species, each represented by one plant, were used. Among them, seventeen woody plants had been grown for more than twenty years on the campus of the Institute. The remaining three grassy plants were grown in upland fields or in paddy fields.

Six extractants were tested, 1) distilled water, 2) 0.5 M Tris-acetate buffer, pH 7.5, 3) buffer solution containing 10% Triton X-100, 4) buffer containing 10% Triton X-100 and 0.5 M sodium chloride, 5) buffer containing 10% Triton X-100 and 0.2 M ascorbic acid, 6) buffer containing 10% Triton X-100, 0.5 M sodium chloride and 0.2 M ascorbic acid. The

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final pH was adjusted with either Tris or acetic acid to 7.5. Fresh green leaves were cut into about 1×1 mm square, crushed and used as leaf samples. One hundred mg of each sample was macerated with 100 mg of quartz sand (200 mesh), 50 mg of polyvinylpyrrolidone and 0.5 ml of each extractant in a mortar and pestle. The slurry was absorbed by a filter paper wick and inserted into a cut made across a starch gel.

The gel buffer consisted of a mixture of 4 mM histidine HCl and 3.6 mM NaOH. The pH value was about 6.7 but changed to about 6.1 after the solution was mixed with 12% hydrolyzed potato starch. The tray buffer consisted of a mixture of 0.5 M trisodium citrate and 4.5 mM citric acid, pH 6.0. Electrophoresis was conducted at 13 v/cm for 3 h and the gel surface was chilled using ice. For the peroxidase determination, the top half slices of the gel were assayed in a reaction mixture consisting of 0.05 M Tris-acetate buffer, pH 4.0, 0.03% hydrogen peroxide, 2 mM 3-amino-9-ethylcarbazole and 2 mM eugenol, the latter two being first dissolved in 10% acetone at the final concentration. MDH zones were detected by immersing the bottom half slices of the gel in a reaction mixture consisting of 0.05 M Tris-acetate buffer, pH 8.0, 0.1 M sodium d,l-malate, 0.06 mM phenazine methosulfate, 0.1 mM NBT and 0.2 mM NAD. The gels were allowed to stand overnight at room temperature in the dark.

The peroxidase zymograms obtained with extractants Nos. 1 to 6 as shown on the left hand side in Figs. 1 and 2, revealed differences in each species. Based on their reactions to the various extractants, the twenty species were classified into five groups as follows: The first group comprised the species in which the six zymograms obtained with the six extractants were nearly identical. This group includes *Sciadopitys*, *Quercus*, *Camellia*, *Cinnamomum*, *Euonymus*, *Nerium*, *Trillium*, *Oryza* and *Howeia*. The second group comprised the species in which appreciable differences were observed, in that the number and intensity of the bands increased with the number of components in the extractants. This group includes *Pteris*, *Cycas*, *Ginkgo*, *Pinus*, *Podocarpus*, *Torreya* and *Prunus*. The third group comprised the species, whose zymograms showed conspicuous changes with the six extractants. The changes were characterized by a remarkable decrease in the number of bands as the number of components in the extractants increased. This group includes *Chamaecyparis* and *Wisteria*. The fourth group comprised the species in which none of the extractants employed were effective in extracting peroxidase. *Aucuba* is a typical species of this group, and *Asarum* also belongs to this group. In addition, a fifth group was identified though the species of this group had already been classified into the four groups mentioned above. Species of this group gave at least one blurred and/or one trailing band in the zymograms obtained with all or most of the extractants. This group includes *Pteris*, *Sciadopitys*, *Howeia*, *Oryza*, *Wisteria*, and *Trillium*, of which the first four gave one blurred band and the last three gave one trailing band.

The MDH zymograms obtained with the six extractants are shown on the right hand side in Figs. 1 and 2, and the twenty species examined were classified into seven groups based on the differences in the reactions of each species to the extractants. The first group comprised the species in which similar zymograms were obtained with the use of any one of the six extractants. This group includes *Torreya* and *Oryza*. The second group comprised the species, whose zymograms were very similar with the use of extrac-

Fig. 1. Peroxidase and MDH starch gel zymograms obtained with six extractants in ten plant species. O shows the startline, top is the anodal and the bottom the cathodal side. The numbers at the bottom represent the kinds of extractants; 1. water, 2. buffer, 3. buffer containing Triton X-100, 4. buffer containing Triton X-100 and NaCl, 5. buffer containing Triton X-100 and ascorbic acid, 6. buffer containing Triton X-100, NaCl and ascorbic acid. Numbers on the left correspond to the names of the following species; 1. *Pteris multifida*, 2. *Cycas revoluta*, 3. *Ginkgo biloba*, 4. *Pinus thunbergii*, 5. *Sciadopitys verticillata*, 6. *Chamaecyparis obtusa*, 7. *Podocarpus macrophyllus*, 8. *Torreya nucifera*, 9. *Quercus sessilifolia*, 10. *Cinnamomum japonicum*. All the zymograms obtained with extractants containing Triton X-100 showed a clear greenish band associated with the presence of decomposed chloroplastic components, near the startline in the cathodal side but the band was omitted here in the drawing.

Fig. 2. Peroxidase and MDH zymograms obtained with six extractants. O shows the startline. The number at the bottom represents the same extractants as those indicated in Fig. 1. The number in the left side indicates the name of the species; 11. *Asarum takaoi*, 12. *Camellia japonica*, 13. *Prunus lannesiana*, 14. *Wisteria floribunda*, 15. *Euonymus japonicus*, 16. *Aucuba japonica*, 17. *Nerium indicum*, 18. *Trillium tschonoskii*, 19. *Oryza sativa*, 20. *Howeia belmoreana*.

tants Nos. 2 to 6 containing a buffer solution. This group includes *Ginkgo*. In the third group, zymograms of the species were almost the same with the use of extractants Nos. 3 to 6, containing a neutral detergent. This group includes *Pinus*, *Sciadopitys*, *Chamaecyparis*, *Podocarpus*, *Quercus* and *Euonymus*. In the fourth group, zymograms of the same with the use of extractants Nos. 5 to 6, containing neutral detergent and antioxidant. This group includes *Cinnamomum*, *Camellia* and *Prunus*. In the fifth group, every extractant was effective in giving bandmorphs, but the zymograms showed appreciable differences, consisting generally of an increase in the number of bands along with the increase in the number of components in the extractants. This group includes *Pteris*, *Cycas*, *Asarum*, *Wisteria*, *Euonymus*, *Nerium*, *Trillium* and *Howeia*. It was noted that extractant No. 4, containing sodium chloride, had a partially inhibitory effect on the intensity of some bands in the zymograms of *Pteris* and *Cycas*. In the sixth group, all extractants appeared to be almost ineffective and gave an extremely weak band or bands in the zymograms. This group includes *Aucuba*. In addition, a seventh group showed one trailing band in the zymograms obtained with extractants containing a neutral detergent. This group includes *Cycas*, *Euonymus* and *Howeia*.

Based on the present experiment, differences in the zymographic pattern of species depending on the extractants employed were revealed in twenty species belonging to twenty families. These findings suggest that intracellular sustaining systems for both enzymes are considerably different among the plant species examined. From this view point, it appears that the sustaining systems of the peroxidase are simpler in the first group which includes *Sciadopitys* and six other species than in other groups. Also, it appears that the sustaining system for both enzymes is the most complicated, i. e., very stable or resistant in *Aucuba*. In taking account of such different systems, more complicated extractants may be required to achieve a better separation of bandmorphs in zymograms. This assumption is valid for the separation of MDH in the species from the first to the fifth groups in contrast with the separation of peroxidase in species such as *Chamaecyparis* and *Wisteria*. The fact that a remarkable decrease in the number of bands was associated with the increase of the number of components in the extractants that a modification of bandmorphs due to hydrolases may occur during the extraction procedure.

Finally the concept of "authentic zymograms" which consist of authentic isozymes is being proposed. It is important to note that it is difficult to define authentic isozymes. As shown in the present experiment, comparatively large blurred bands as well as trailing bands near the startline were detected in eight species. However the presence of such bands was disregarded since the number of isozyme bands could not be determined accurately. The following definition of isozymes can thus be proposed; when the number of isozyme bands and their intensities in the zymograms increase with the incorporation of neutral buffer, detergent, salt and antioxidant in the extractants, all the newly detected isozyme bands are considered to be authentic, irrespective of *in vivo* modification of their enzyme molecules. In contrast, when the number of isozyme bands decreases with the number of components in the extractants, the lack of definition of the bands can be attributed to *in vitro* modification due to the extraction of localized modifier enzymes and the isozyme bands can not be considered to be authentic.

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20 植物種の緑葉パーオキシダーゼおよびリンゴ酸脱水素酵素における 抽出剤依存性ザイモグラムに関するノート

遠藤 徹・井原正昭

(国立遺伝学研究所 育種遺伝部, 三島市, 〒 411)

ゲルを用いるアイソザイム検出に際し, 緑葉に対して用いる抽出剤の組成としての緩衝液 (0.5M トリス-酢酸 pH 7.5), 中性洗剤 (10% トリトン X-100), 塩 (0.5M 食塩), 酸化防止剤 (0.2M アスコルビン酸) およびフェノール吸収剤 (ポリビニールポリピロリドン) について, その単独および複合効果を調査した. 用いた材料はシダ植物 (イノモトソウ) 裸子植物 (ソテツ, イチョウ, クロマツ, コウヤマキ, ヒノキ, イヌマキ, カヤ), 双子葉植物 (ツクバネガシ, ヤブニッケイ, ヒメカンアオイ, ツバキ, オオシマザクラ, フジ, マサキ, アオキ, キョウチクトウ) および単子葉植物 (ミヤマエンレイソウ, ニホンイネ, ケンチャヤシ) の生葉 100 mg で, ザイモグラムとして検出した酵素種はパーオキシダーゼとリンゴ酸脱水素酵素である.

パーオキシダーゼの場合, 6種の抽出剤に対して少なくとも4群に分類できる. 第1群はコウヤマキなどの抽出剤でも同じようなザイモグラムが得られるもの, 第2群は抽出剤成分の種類の増加につれてアイソザイムバンド数が増加するもの, 第3群は逆にバンド数が減少するもの, 第4群はアオキで6種の抽出剤のいずれを用いても抽出が極めて困難なものなどである. リンゴ酸脱水素酵素の場合は6群に分類できる. 第1群はカヤとイネの2種である. 第2群はイチョウ1種だが緩衝液を含む抽出剤を用いれば同じようなザイモグラムが得られる. 第3群はクロマツなどで中性洗剤を含む緩衝液の抽出剤では同じようなザイモグラムが得られる. 第4群は中性洗剤と酸化防止剤を含む緩衝液の抽出剤で同じようなザイモグラムが得られる. 第5群は抽出剤の成分数の増加に応じて一般にバンドが増加するもの, 第6群はパーオキシダーゼの場合と同じくアオキで, 用いた抽出剤の組成ではほとんど抽出困難な場合である.

以上の結果から, 生体内におけるアイソザイムの存在様式ないし保持機構は植物種ごとに多かれ少なかれ異なり, 例えば水でほとんど全部を抽出し得る場合から上記のすべての薬剤を投入しても抽出困難な場合まである. すなわち, アイソザイムにおける分化と同様, その保持機構もまた分化していると推論できる.