Intracellular Localization and Molecular Forms of Phenylalanine Ammonia-Lyase in Apple Fruit (*Pyrus Domestica* Borkh.)

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ABSTRACT

The present study is a first report describing the intracellular localization and isoenzyme composition of phenylalanine ammonia-lyase in "Gizil Ahmedy" and "Renet Simirenko" varieties of apple fruit (*Pyrus Domestica* Borkh.). It was determined, that the greater part of the total enzyme activity (76.5% and 80.1% respectively) in apple subepidermal tissues was found in the cytoplasmic fraction, and small amount of activity (6.6% and 5.7%, respectively) was found in 105 000g pellet. Polyacrylamide disk gel electrophoresis revealed two molecular forms of the enzyme with significantly different electrophoretic mobilities. The most active and dominant molecular form of phenylalanine ammonia-lyase was present in the cytosol, and less active form was found in subcellular particles precipitating at 105 000g. This investigation allowed to demonstrate the cellular compartments assosiated with the anabolism of phenolic compounds, that have a great interest for the regulation of postharvest life procesess in apple fruits.

Keywords: phenylalanine ammonia-lyase (PAL), PAL activity, molecular forms of PAL, intacellular localisation of PAL, apple fruit, *Pyrus Domestica*.

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INTRODUCTION

Phenylalanine ammonia-lyase (PAL; E.C.4.3.1.5) catalyses the reversible conversion of L-phenylalanine (L-Phe) to trans-cinnamic acid (*t*-CA) and ammonia. It is the first and key enzyme of the secondary metabolic pathway, so-called phenylpropanoid sequence, which operative in higher plants and is mainly involved in defense mechanisms [1- 5].

PAL is absent in true bacteria and animal tissues [6]. It has been relatively well studied in few microorganisms, which use L-Phe as a sole source of carbon and nitrogen [7, 8], in yeasts [9 – 12] and in some fungi [13]. However, few studies have been carried out in plant tissues [1, 14], especially sap-containing fruits [3, 4].

It was demonstrated, that PAL frequently consists of several molecular forms and their quantities can vary depending on the tissue and species, grows and developmental stages, as well the growth conditions. In addition, these molecular forms can be present in different parts of the cell and can have significantly different properties [11, 15 – 18].

PAL is a tetrameric enzyme whose subunits are encoded by multigene families in most species studied [19 – 22]. In tobacco (*Nicotiana tabacum*) PAL genes have a simple organization consisting of two families, each with two very closely related genes [20, 23].

In the previous study we performed optimization of methods for extraction of PAL from apple subepidermal tissues and for evaluation of its activity; as well we described some properties of this enzyme [24]. In the present research we continue our previous investigations.

The objectives of our present study were to determine the intracellular localization and isoenzyme composition of PAL in apple fruit.

MATERIALS AND METHODS

Plant material

Experiments were carried out using two apple varieties: "Gizil Ahmedy" and "Renet Simirenko". Despite the fact, that both of them belong to the winter varieties, their fruits differ significantly in some properties. Thus, "Gizil Ahmedy" is a southern, thermophilic apple variety and has a stronger pigmentation. The skin of ripened fruits has dark red color. The ripened fruits of "Renet Simirenko" variety have green color. This apple variety grows in northern regions and more cold resistant. Prior to use, fruits were stored in the refrigerator at 4 $^{\circ}$C.

Enzyme extraction

Enzyme extraction and assay for activity were performed as described previously [24]. Apple fruits subepidermal tissue (10 g) were ground with 20 ml of extraction buffer containing 20 mM tris-HCl pH 8.5, 1% Polyclar-AT (insoluble Polyvinylpyrrolidone) and 0.01 M 2-mercaptoetanol. Homogenate were filtered through nylon gauze, and the resulting filtrates were sequentially centrifuged at 1000g, 10 000g, 30 000g and 105 000g. Enzyme activity and isoenzyme composition were studied directly in supernatants, and also in pellets, after treatment with 0.1% Tween-80 in extraction buffer. In some experiments, in order to preserve subcellular structural organization, the osmotic shock was prevented by adding 0.5 M sucrose to the extraction buffer. Ionic strength was maintained using 0.5 M KCl, which was added to the supernatant or the extraction buffer, depending on experiment.

Assay of enzyme activity

PAL activity was measured spectrophotometrically at 290 nm in terms of the rate of *t*-CA formation. Incubations were carried out in 0.15 M tris-HCl buffer pH 8.5, containing 0.12 M L-Phe and 0.5 ml of enzyme preparation in a final volume of 3 ml. Controls were identical but lacked substrate. Enzyme activity was expressed in nM of *t*-CA formed per min per g of tissue. All experiments were performed in triplicate. Protein concentration was determined by the Bradford method [25].

Polyacrylamide disk gel electrophoresis

Polyacrylamide disk gel electrophoresis (Reanal, Hungary) was carried out in alcaline buffer system according to Davis (1964) [26]. Microporous gels were prepared using 7.5% acrylamide, and macroporous gels using 5.0% acrylamide; 25.0% sucrose was added to prevent convection. Electrophoresis was carried out for 3 h at 4 $^{\circ}$C. The current was 1 mA for the first 20 min and then 3 mA per tube. Enzyme bands were detected by incubating gels in the same medium used for enzyme assay, supplemented with 0.005 mg/ml phenazine metosulfate and 0.05 mg/ml *p*-iodnitroblue tetrazolium; incubations were carried out for 4 h at 30 $^{\circ}$C in the dark.

RESULTS

Intracellular localization of PAL activity

Most part of enzyme activity was assosiated with the supernatant. Thus, for example, the 105 000g supernatants from "Gizil Ahmedy" and "Renet Simirenko" apples contained about 76.5% and 80.1% of the total enzyme activity, respectively. Small amounts of activity (6.6% and 5.7% for "Gizil Ahmedy" and "Renet Simirenko", respectively) were found in 105 000g pellet after treatment with Tween-80 in the presence of KCl. In 10 000g and 30 000g pellets the PAL activity was not found (Table 1).

Table 1: Distribution of PAL activity in subcellular fractions of apples (nmoles *t*-CA/min/g of tissue)•

Fractions	Gizil Ahmedy	Renet Simirenko
Supernatant 1000g	187 (88.3)	141 (90.4)
Supernatant 105000g	162 (76.5)	125 (80.1)
Pellet 10000g	0	0
Pellet 30000g	0	0
Pellet 105000g	16 (6.6)	9 (5.7)

•numbers in bracets show activity as a persentage of the total.

The effects of extraction buffer (EB) composition on PAL activity in different supernatants from apples shown in Table 2. The most active was the 1000g supernatant prepared

using extraction buffer lacking KCl and sucrose. Activity in 10 000g and 30 000g supernatants was similar to what was observed in previous supernatant (1000g). However, further centrifugation resulted in remarkable reduction in PAL activity in 105 000g supernatant. Addition of KCl to the extraction buffer reduced total activity in 1000g supernatant by 13.0% and 9.0% in "Gizil Ahmady" and "Renet Simirenko" apples, respectively. The use of KCl together with sucrose reduced activity in 1000g supernatant by 5.8%.

Table 2: Effects of the extraction buffer (EB) composition on PAL activity in different supernatants from apples (nmoles *t*-CA/min/g of tissue)

Superna-tant	Gizil Ahmedy			Renet Simirenko		
	extraction buffer (EB)	EB+KCl	EB+KCl+ sucrose	extraction buffer (EB)	EB+KCl	EB+KCl+ sucrose
1000g	192±2.4	180±2.1	171±2.1	150±1.8	133±1.9	121±1.2
10000g	190±1.3	172±2.5	166±2.4	148±1.3	121±1.4	113±1.3
30000g	187±3.4	169±3.1	164±2.5	146±2.6	118±3.1	111±2.1
105000g	160±1.1	160±2.7	158±3.0	120±2.1	113±2.2	107±1.8

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05)

Molecular forms of PAL

Figure 1 shows zymograms of the isoenzyme composition of different fractions of apple subepidermal tissues, which demonstrate the number of molecular forms of PAL and their intracellular localisations. Zymograms of 1000g, 10 000g and 30 000g supernatants were similar, as well of 1000g, 10 000g and 30 000g pellets.

Electrophoretic analysis of 1000g, 10 000g and 30 000g supernatants resulted in detection of two molecular forms of the enzyme with significantly different electrophoretic mobilities (gel N1). These forms had Rf values of 0.14 and 0.35. Electrophoretic analysis of 105 000g supernatants prepared in the presence of 0.5 M sucrose yelded a single molecular form of the enzyme with Rf 0.14 (gel N2). The other form, with Rf 0.35 was not found.

The gel N3 showes electrophoretic analysis of the pellet from the 105 000g supernatant after treatment with Tween-80. In this gel, the region corresponding to Rf 0.35 showed typical staining, while the Rf 0.14 band had disappeared. The 10 000g and 30 000g pellets showed no enzyme activity (Fig.1).

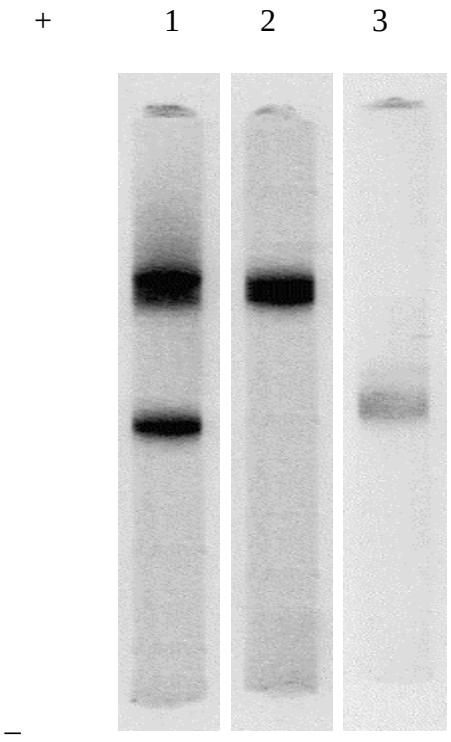

Fig. 1: Disk electrophoresis of different fractions from subepidermal tissue of "Gizil Ahmedy" apples:

1 – 1000g supernatant;

2 – 105000g supernatant;

3 – 105000g pellet treated with Tween-80

Discussion

The enzyme distribution from different apple tissue fractions showed that different levels of PAL activity present in different tissues. More than 70.0% of enzyme activity was determined in 105 000g supernatants, but only 6.0% of activity (6.6% and 5.7% for "Gizil Ahmedy" and "Renet Simirenko", respectively) were found in 105 000g pellet. This enzyme distribution suggests that the cytoplasmic form of PAL was predominant in subepidermal apple tissue. Our results agree with exsisting data which show that plant PAL is predominantly cytosolic [27 - 31]

As already noted, subcellular particles of apple cells that was pelleted at 105 000g, had low activity. However, it is still possible that the real level of enzyme activity in this pellet was much higher, because treatment with Tween-80 and KCl could itself reduce activity. In addition, the optimal conditions for PAL activity in subcellular particles might differ from those obtained for the cytosolic form (Table 1).

It is interesting to note that the extraction medium composition had significant effect on the level of PAL activity and the way it changed in supernatants produced by different centrifugation conditions. Loss of activity after centrifugation at 105 000g of the most active 1000g supernatant suggests that some proportion of enzyme is bound to subcellular particles.

Despite the fact that addition of KCl to the extraction buffer reduced total activity in 1000g supernatant, loss of activity in subcequent supernatant was prevented. The ionic strength resulted from extraction buffer, so that the pelleting of particles by centrifugation would no longer be reflected by the level of activity remaining in the supernatant. The reduction in total PAL activity after addition of KCl was probably associated with its direct action on enzyme, as the same effect was seen when KCl was added to the incubation mix for enzyme assay.

A sucrose had no effected on *in vitro* PAL activity, but simultaneous addition of KCl reduced activity in 1000g supernatant. Thus, the osmotic pressure created by sucrose addition and resulted protection of subcellular particles prevented the release of enzyme from the particles into the extraction buffer. Thus, experiments carried out in the presence of sucrose also did not show differences between supernatant activities after removal of subcellular particles by centrifugation. Comparative analysis of all three extraction buffer variants suggested, that 0.5 M KCl released the same proportion of PAL activity that is associated with subcellular particles which was damaged during preparation of supernatants. It is known, that PAL is an operationally soluble enzyme, that does not possess any obvious membrane anchor or membrane-spanning domains. Nevertheless, biochemical fractionation studies revealed that endoplasmyc reticulum (ER) provide an alternative location for PAL in plant tissues, where it associated with ER membranes [14, 15, 17, 28, 30, 32]. Some authors reported, that PAL and other flavonoid pathway enzymes in buckwheat (*Fagopyrum esculentum*) were loosely associated with the cytoplasmic face of the ER [33]. An observation that we made in our study indicates that the enzyme is also present in ER. Thus, PAL activity was always detected in microsomal fraction pelleted at 105 000g and the pellet of this fraction contained particles of ER.

Thus, most of the PAL activity in apple fruits was located in the cytoplasmic fraction, with smaller quantities appearing in the subcellular particles pelleting at 105000g. In the study of Rasmussen and Dixon (1999) [29], a significant percentage of PAL activity in the wild-type tobacco was detected in microsomal fraction, as well was present in cytosol.

The next series of experiments were devoted for analysis of the isoenzyme composition of different fractions of apple subepidermal tissues. Electrophoretic analysis of 1000g, 10 000g and 30 000g supernatants resulted in detection of two molecular forms of enzyme with significantly different electrophoretic mobilities and *p*-iodnitroblue tetrazolium staining intensities (gel N1, Fig. 1). The isoenzyme band with Rf 0.14 stained more intensely and it was significantly wider. This would thus appear to be the dominant molecular form of PAL in apple fruit.

Electrophoretic analysis of 105 000g supernatants gave a single molecular form of PAL with Rf 0.14 and without isoenzyme with Rf 0.35 (gel N2). This show that the dominant molecular form with Rf 0.35 is present in the cytoplasm, while the other is located in subcellular particles pelleting at 105 000g. This was also supported by electrophoretic analysis of the pellet from the 105 000g supernatant after treatment with Tween-80 (gel N3).

Electrophoretic studies of "Renet Simirenko" apples gave similar results. Two molecular forms were observed, one of which was present in subcellular particles, while the second, dominant form, was located in the cytosol. The only difference was that the band with Rf 0.14 stained less intensely than their counterparts in "Gizil Ahmady" apples, and this correlated with the different activities in the appropriate fraction of two apple varieties (Table 1). Thus, the subepidermal tissue of apple fruit contains most of the PAL activity in the cytosol with a smaller quantity in subcellular particles precipitating at 105000g. Each of fractions consists of a single form of PAL with significantly different electrophoretic mobilities.

CONCLUSION

It was determined, that the greater part of the total enzyme activity (76.5% and 80.1% respectively) in apple subepidermal tissues was found in the cytoplasmic fraction, and small amount of activity (6.6% and 5.7%, respectively) was found in 105 000g pellet. Polyacrylamide disk gel electrophoresis revealed two molecular forms of the enzyme with significantly different electrophoretic mobilities. The most active and dominant molecular form of phenylalanine ammonia-lyase was present in the cytosol, and less active form was found in subcellular particles precipitating at 105 000g.

This investigation allowed to demonstrate the cellular compartments assosiated with the anabolism of phenolic compounds, that have a great interest for the regulation of postharvest life procesess in apple fruits.

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