Influence of axis removal on proteolytic activities after imbibition in *Citrus* seeds. Characterization of aminopeptidase activity

Leonor Lapeña
Pilar García-Agustín*

Unidad de Biotecnología Vegetal,
Dpto. Ciencias Experimentales,
Universitat Jaume I, Aptd. 224,
12080 Castellón, Spain

Influence of axis removal on proteolytic activities after imbibition in *Citrus* seeds. Characterization of aminopeptidase activity.

Abstract — Introduction. The study of the regulation of the proteolysis in the germination process of *Citrus* seeds would help to improve the quality of the seeds from the agronomic point of view. Storage proteins are mobilized by different proteolytic enzymes such as endo-, amino- and carboxypeptidases in cotyledons of different species. The removal of the embryonic axis affected proteolytic activities. Several works showed that the effect of the axis may be replaceable by hormones. **Materials and methods**. Monoembryonic seeds of *Citrus limon* L. Burn. f. (cv Fino) were used in all experiments. After 2, 4 and 5 d after imbibition, cotyledons were homogenized and used for the determination of proteolytic activities and electrophoretic analysis. **Results and discussion**. In *Citrus* cotyledons, the axis removal accelerated the proteolysis at the beginning of imbibition. When cotyledons were detached before the onset of imbibition, the carboxy- and endopeptidase activities were strongly depressed. In contrast, aminopeptidase activity increased to a higher level than that of the attached one. The effect of the axis on endopeptidase activity was replaceable by kinetin. **Conclusion**. Aminopeptidase activity may be the cause of the acceleration of proteolysis at the beginning of imbibition. This activity had at least three bands in the cotyledons. Endo-, carboxy- and aminopeptidases may be sulfhydryl peptidases or serine proteases. (© Elsevier, Paris)

***Citrus* / germination / seeds / enzymic activity**

Influence de l'ablation de l'axe embryonnaire sur les activités protéolytiques de la graine d'agrume, après imbibition. Caractérisation de l'activité de aminopeptidase.

Résumé — Introduction. L'étude de la régulation des protéolyses au cours de la germination des graines d'agrumes pourrait aider à améliorer la qualité agronomique des graines. Dans les cotylédons de nombreuses espèces, le stockage des protéines est mobilisé par différents enzymes protéolytiques tels que les endo-, amino- et carboxypeptidases. L'ablation de l'axe embryonnaire affecte les activités protéolytiques. Plusieurs travaux ont montré que l'effet de l'axe peut être remplacé par des hormones. **Matériel et méthodes**. Des graines monoembryonnées de *Citrus limon* L. Burn. f. (cv. Fino) ont été utilisées pour chacune des expérimentations. Après 2, 4 et 5 d d'imbibition, les cotylédons ont été broyés, homogénéisés, puis utilisés pour évaluer les activités protéolytiques et effectuer leur analyse électrophorétique. **Résultats et discussion**. L'ablation de l'axe a accéléré les protéolyses au début de l'imbibition des cotylédons. Dans les cotylédons séparés de leur axe avant imbibition, les activités carboxy- et endopeptidases ont été fortement abaissées. Au contraire, l'activité aminopeptidase a augmenté jusqu'à dépasser le niveau d'activité observé chez les cotylédons non séparés. L'effet de l'axe sur l'activité aminopeptidase a pu être remplacé par de la kinétine. **Conclusion**. L'activité aminopeptidase pourrait être à l'origine de l'accélération des protéolyses observée au début de l'imbibition. Cette activité a présenté au moins trois bandes lors de l'électrophorèse des cotylédons. Les endo-, carboxy- et aminopeptidases pourraient être des peptidases des sulfhydriles ou des protéases de la sérine. (© Elsevier, Paris)

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* *Correspondence and reprints*

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1. introduction

Little information on the regulation of the proteolysis in the germination process of *Citrus* seeds is available [1], so it would be of great interest to study in detail the mechanistic aspects of regulation of this process. This would eventually help to improve the quality of the seeds from the agronomic point of view.

Citrus seeds contain large amounts of storage proteins in the cotyledons [2]. During germination, different proteolytic activities (endo-, carboxy- and aminopeptidase) have been observed in *Citrus* cotyledons [1]. Proteolytic enzymes such as endo-, amino- and carboxypeptidases have been studied in different dicotyledonous species [3–7]. There is evidence that in certain mono- and dicotyledonous species the removal of the embryonic axis depresses proteolytic activity [8–14] and, consequently, embryonic axis plays a role in the control of the protein breakdown [1, 11, 12, 15–17]. Recently, Mullen and Gifford [18] supported the involvement of the embryonic axis in regulating lipid mobilization in castor bean seeds.

On the other hand, Gifford et al. [19] showed that the effect of the embryonic axis could be replaced by gibberellins in castor bean. In detached cotyledons of squash [8] and in germinating beans [20], the effect of the axis on protease activity was replaceable by cytokinins. In contrast, the mobilization of proteins could not be regulated by hormones in *Vigna* species [14].

García-Agustín et al. [1] demonstrated the effect of the axis during germination (from 8 to 40 days) on different proteolytic activities in *Citrus*, but more information was required to determine possible mechanisms regulating the cotyledonary proteolytic activity at the beginning of the germination.

The objective of this work was to study the effect of the axis on proteolytic activities in *Citrus* seeds, several days after imbibition and to characterize the aminopeptidase protein.

2. materials and methods

2.1. sample preparation

Monoembryonic seeds of *Citrus limon* L. Burm. f. (cv. Fino) were used in all experiments. Seeds were sterilized in 2% commercial bleach for 20 min after removal of the seed coats. Whole seeds and detached cotyledons were placed on layers of wet filter paper at 27 °C in petri dishes containing either 10 mL of distilled water or 10 mL of 10^{-4} M kinetin, gibberellic acid (GA) or abscisic acid (ABA).

One g of fresh weight from cotyledons was homogenized with a mortar, 2, 4 and 5 days after imbibition, and pestled at 4 °C in 4 mL of 50 mM Tris-HCl (pH 7.5) containing 10 mM 2-mercaptoethanol. Homogenates were centrifuged at 27 000 *g* for 20 min at 4 °C and the supernatants were used for the determination of aminopeptidase, carboxypeptidase and endopeptidase activities, for preparing the samples for electrophoresis and for assaying protein content. Analytical values are the means of three different experiments.

2.2. protein analysis

Protein was measured according to the Bradford method [21], using BSA (bovine serum albumin) as a standard. One hundred µL of the supernatant solution was added to 5 mL of Bradford reagent (100 mg of Coomassie G blue in 50 mL ethanol 96%, adding 100 mL of H_3PO_4 and up to 200 mL of distilled water). The absorbance of all samples was read at 595 nm.

2.3. enzyme activity measurements

Endopeptidase (EP) activity was measured using azocasein (10 $mg \cdot mL^{-1}$) as substrate [22]. Two-hundred µL of extract was incubated with 300 µL of substrate solution, 20 mM 2-mercaptoethanol and 500 µL of 250 mM sodium-acetate buffer (pH 4.5) or Tris-HCl buf-

fer (pH 7.5). The mixture was incubated for 4 h at 40 °C and the reaction was stopped by adding 2 mL of 10 % trichloroacetic acid (TCA) solution. After 45 min at 4 °C, the solid suspension powder in the stopped mixture was removed by centrifugation at 1600 *g* for 10 min, and the absorbance monitored at 340 nm. One unit of enzyme activity was defined as the increase of 0.01 absorbance unit·g^{-1} of fresh weight·h^{-1} under the assay conditions. Data were compared to zero-time blanks in all experiments.

Carboxypeptidase (CP) activity was determined with N-carbobenzoxy-L phenylalanine-L alanine (CBPA) [3]. CBPA was dissolved in warm dimethylsulfoxide (37 mg·mL^{-1}) and made up to a total volume of 50 mL with 50 mM Na_2HPO_4-citrate buffer (pH 5). The final concentration of the substrate was 2 mM. Two mL of this solution was incubated with 200 µL of extract for 2 h at 30 °C. The reaction was stopped with 1 mL of 15% TCA. The proteins were allowed to precipitate for 15 min at 4 °C (10 min at 1600 g). The amino acid content of the supernatant was determined using the ninhydrin method [23]. One unit of activity was defined as 1 µg of amino acid released·g^{-1} fresh weight·h^{-1} under the assay conditions. Data were compared to zero-time blanks in all experiments.

The aminopeptidase (AP) activity was determined by measuring the increase in absorbance at 410 nm after incubation of the extracts with L-alanine-p-nitroanilide (Ala-pNA) as substrate [22]. For spectrophotometric determination, the substrate (1 mg·mL^{-1}) was dissolved in Tris-HCl (pH 7.5). Six-hundred µL of this solution were mixed with 200 µL of the extract and 600 µL Tris-HCl (pH 7.5), and incubated at 40 °C for 30 min. The reaction was stopped by placing the reaction tubes on a steam bath for 5 min. The absorbance was measured after addition of 1.2 mL of distilled water. One unit of enzyme activity was defined as the increase of one absorbance unit·g^{-1} fresh weight·h^{-1}. Data were compared to zero-time blanks in all experiments.

The inhibition of the EP, CP and AP activities were assayed by adding leupeptin (10 µM), PMSF (phenylmethylsufonyl fluoride 2 mM) and EDTA (ethylenediaminetetraacetic acid 5 mM) to the extracts before incubation with the substrates and determining its activity.

An electrophoretic analysis of the amimopeptidase activity was carried out with samples prepared by adding 43 µL of a solution containing 0.2 M Tris-HCl (pH 8.3), and 25% (v/v) glycerol to 100 µL of extract. Molecular mass markers included: bovine serum albumin (BSA), 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; α-lactalbumin, 14 kDa (Sigma). Samples were electrophoresed on non-denaturing 12% polyacrylamide gels at constant voltage, first 70 V for 30 min and then 150 V for 2 h. After electrophoresis, the gels were incubated for 1 h at 37 °C with a substrate solution containing L-alanine-β-naphtylamide (Ala-β-NA) at 0.5 mM in 250 mM Tris-HCl (pH 7.5) and stained with Fast Black K salt dissolved in 0.1 M acetate-NaOH buffer (pH 4.2) [22].

3. results

In attached cotyledons, the protein content was progressively reduced during the first days after imbibition. When cotyledons were detached from the axis, the proteolysis was accelerated until 5 d after imbition (*figure 1*).

All three enzyme activities were found in dry cotyledons and their levels increased after imbibition with water (*figure 2*).

In attached cotyledons, endopeptidase (azocaseolytic enzyme) activity increased rapidly and reached a maximum level 5 d after imbibition (*figure 2a*), carboxypeptidase activity showed a peak at the second day (*figure 2b*) and aminopeptidase activity increased after 2 d of imbibition, then it was constant (*figure 2c*).

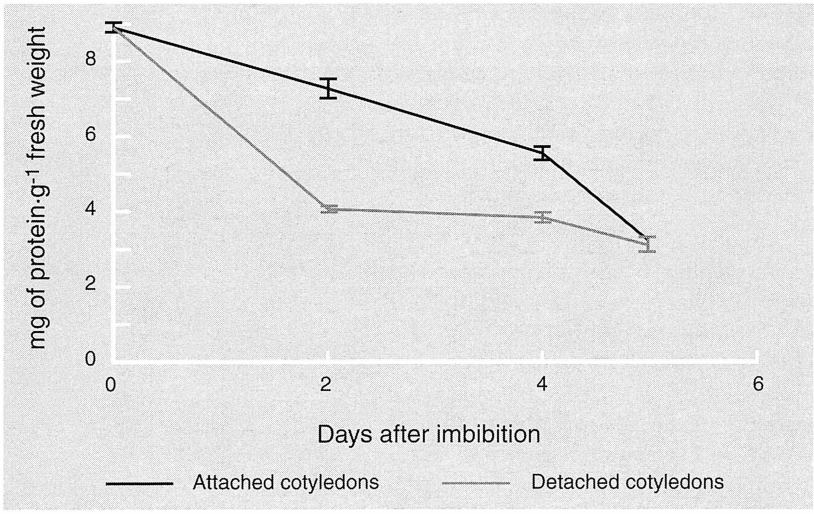

Figure 1.
Effect of axis removal on protein content in cotyledons of *Citrus limon* seeds during the first days after imbibition.

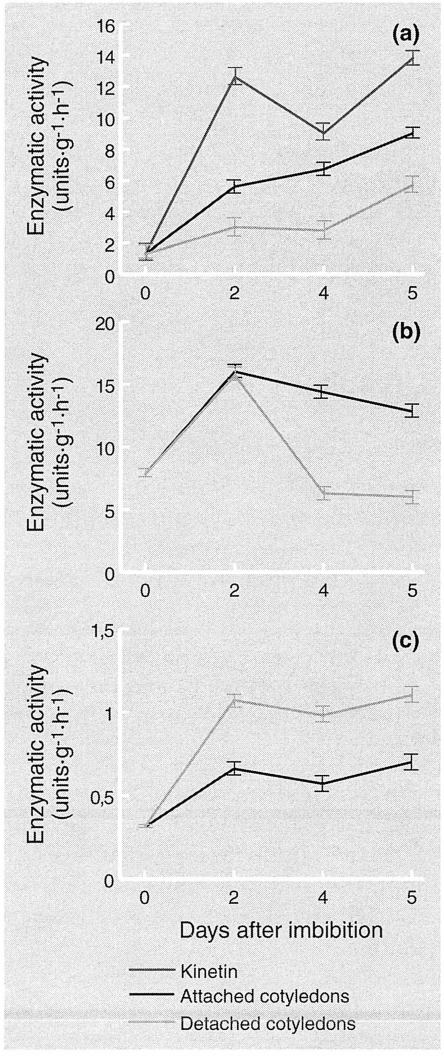

Figure 2.
Changes in enzymatic activity in kinetin-treated, attached and detached cotyledons of *Citrus limon* during the first days after imbibition.
a. Endopeptidase activity;
b. Carboxypeptidase activity;
c. Aminopeptidase activity.

When cotyledons were detached from germinating seeds, the endopeptidase and carboxypeptidase activities were strongly depressed but the aminopeptidase activity increased to a higher level than that of the attached ones (*figure 2a, b, c*). In order to examine the possibility that these enzymatic activities may be stimulated by plant hormones produced in the axis, detached cotyledons were incubated with solutions of kinetin, GA and ABA (10^{-4} M) and the endo-, carboxy- and aminopeptidase activities were determined after the incubation. In detached cotyledons, endopeptidase activity increased during incubation with kinetin (10^{-4} M) (*figure 2a*). No significant effects were observed in amino- and carboxypeptidase activities during incubation with any plant hormones assayed (kinetin, GA and ABA).

After polyacrylamide gel electrophoresis of attached and detached cotyledons extracts, aminopeptidase activity had at least three bands (*figure 3*). The apparent molecular mass of bands was approximately 70–56 kDa. The intensity of the bands increased during the first days after imbibition (2, 4 and 5 d) and is more prominent in detached than in attached cotyledons (*figure 3*).

Aminopeptidase activity was inhibited by leupeptin (sulfhydryl protease inhibitor) and PMSF (serine-protease inhibitor) at 70% and 40% respectively, whereas this activity was inhibited only at 5% by the chelating agent (*table I*).

Carboxypeptidase activity, using CBPA as substrate, was 97% inhibited by PMSF and 54% by leupeptin. Endopeptidase activity was inhibited by sulfhydryl protease inhibitor (61%), by the chelating agent (58%) and by PMSF (81%) (*table I*).

4. discussion

Previous works showed that, in *Citrus* cotyledons, after 8 d of germination, the axis removal accelerated the

proteolysis [1]. In this paper, similar results have been found, for 0 to 5 d after imbibition, when cotyledons were detached from the axis.

Several works had shown that the proteolytic enzymes (endo- and exopeptidases) were involved in the degradation of storage proteins in cotyledons of germinating seeds [1, 3–5, 7, 14, 24].

The depression of proteolytic activity by axis removal was reported in squash [8], pea and *Vigna* species [9–13]). In contrast, Mitsuhashi et al. [14] found, in *Vigna mungo* seeds, that the carboxypeptidase activity increased in detached cotyledons; García-Agustín et al. [1] found similar results in *Citrus* cotyledons, 12 d after germination. However, this work showed that, in the first days of imbibition of detached *Citrus* cotyledons, a decrease in the carboxypeptidase activity was produced. This result may indicate that, at the onset of imbibition, this activity is associated with the mobilization of storage protein, whereas 8 d after germination it is associated with the senescence of cotyledons [1]. Moreover, aminopeptidase activity increases in detached cotyledons, which may be the cause of the acceleration of proteolysis at the beginning of imbibition.

Previous results showed that the embryonic axis controls food mobilization in dicotyledonous seeds by two possible mechanisms: a) hormonal control; and b) sink effects. In the first mechanism, the effect of the embryonic axis may be replaceable by growth regulators. Many studies have been performed on hormonal control [1, 8, 20, 25].

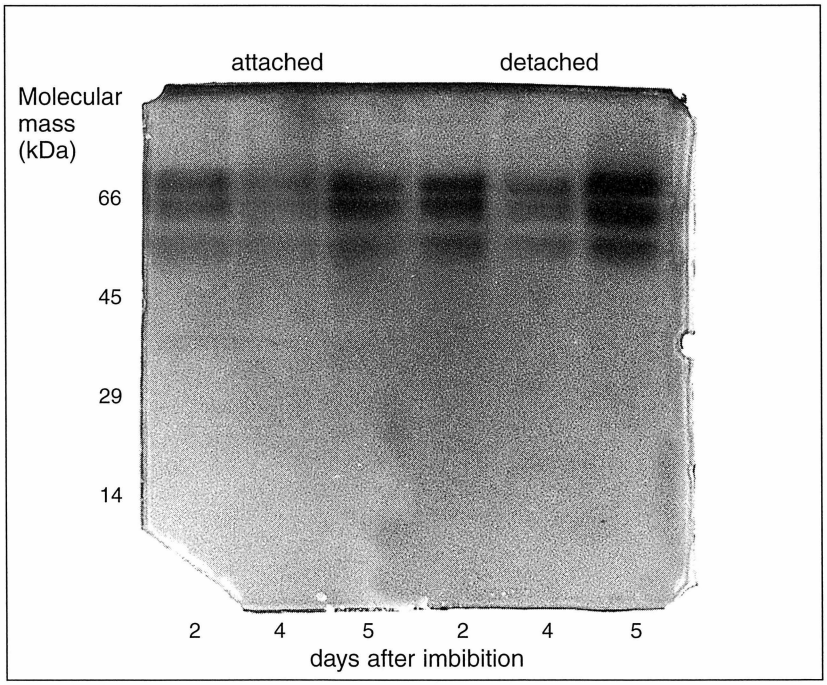

Figure 3.
Electrophoresis of extracts from attached and detached cotyledons of *Citrus limon*, 2, 4 and 5 d after imbibition. Molecular mass (kDa) markers are shown on the left.

The present study shows that the endopeptidase activity in *Citrus* cotyledons may be restored by exogenous kinetin but not the amino- and carboxypeptidase activities (data not shown). This fact suggests that hormones may

Table I.
Effect of inhibitors on proteolytic activities of citrus seeds extracts after 4 days of imbibition. All analytical values are the means of three different experiments. Leupeptin is a sulfhydryl protease inhibitor, PMSF (phenylmethylsulfonyl fluoride) is a serine-protease inhibitor, and EDTA (ethylenediaminetetraacetic acid) is a chelating agent.

Inhibitor	EP activity		CP activity		AP activity	
	Units of activity	Inhibition (%)	Units of activity	Inhibition (%)	Units of activity	Inhibition (%)
Control	6.70 ± 0.71	0	14.34 ± 1.00	0	0.57 ± 0.09	0
Leupeptin (10 µM)	2.60 ± 0.42	61	6.55 ± 1.26	54	0.17 ± 0.02	70
PMSF (2 mM)	1.27 ± 0.36	81	0.43 ± 0.12	97	0.34 ± 0.15	40
EDTA (5 mM)	2.81 ± 0.75	58	11.10 ± 2.56	22	0.54 ± 0.06	5

regulate the development of endopeptidase activity, but not the development of amino- and carboxypeptidase activities.

Several aminopeptidases activities have been isolated from cotyledons of *Vigna* species [7, 26]. In our work, at least three bands of aminopeptidase activity were found in attached and detached *Citrus* cotyledons.

Endopeptidase activity assayed on azocasein was sensitive to inhibitors of sulfhydryl peptidases and serine-proteases (*table I*). Previously, a sulfhydryl endopeptidase was found in legume seeds [27, 28] and a serine endopeptidase was found in *Vigna mungo* seeds [24]. The sensitivity of aminopeptidase activity to leupeptin seems to indicate that this activity may be constituted by a sulfhydrylpeptidases, although aminopeptidase activity was sensitive also to PMSF inhibitor. Recently, in mung bean cotyledons, Yamaoka et al. [7] showed that aminopeptidase requires free sulfhydryl for its activity. The inhibition of carboxypeptidase activity in a 97% by PMSF may indicate that this protein is a serine protease.

5. conclusion

Aminopeptidase activity increases in detached *Citrus* cotyledons which may be the cause of the acceleration of proteolysis at the beggining of imbibition. Exogenous kinetin may restored endopeptidase activity in *Citrus* axis removal cotyledons. Aminopeptidase activity had at least three bands in attached and detached cotyledons. The sensitivity to different inhibitors of endo-, carboxy- and aminopeptidases seems to indicate that these proteins may be sulfhydryl peptidases or serine proteases.

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Influencia de la separación del eje embrionario sobre las actividades proteolíticas tras la imbibición de semillas de cítricos. Caracterización de la actividad aminopeptidisa.

Resumen — Introducción. Estudiar la regulación del proceso proteolítico de las semillas de cítricos durante la germinación, podría ayudar desde el punto de vista agronómico a mejorar la calidad de las semillas. En los cotiledones de varias especies, las proteínas de reserva se mobilizan por diferentes actividades proteolíticas tales como endo-, amino- y carboxipeptidasa. La eliminación del eje embriónico afecta a las actividades proteolíticas. Varios trabajos han mostrado que el eje embriónico puede ser reemplazado por hormonas. **Material y métodos**. Semillas monoembriónicas de *Citrus limon* L. Burm. F. (cv. Fino) se utilizaron en todos los experimentos. Después de 2, 4 y 5 d después de la imbibición, los cotiledones se homogeneizaron y se centrifugaron, el extracto obtenido se utilizó para analyzar las actividades proteolíticas y para realizar la electroforesis. **Resultados y discusión**. En cotiledones de cítricos, la separación del eje embriónico acelera la proteólisis al inicio de la imbibición. Cuando los cotiledones se separaban del eje embriónico antes del inicio de la imbibición, se observó que las actividades carboxi- y endopeptidasa se inhibían. En contraste, la actividad aminopeptidasa era superior cuando se compararon con cotiledones unidos eje. La actividad endopeptidasa era reemplazada por kinetina cuando se eliminaba el eje embriónico. **Conclusión**. La actividad aminopeptidasa parece ser la responsable de la proteólisis al inicio de la imbibición. Dicha actividad presenta al menos tres bandas en la electroforesis de todos los cotiledones. La actividad endo-, carboxi- y aminopeptidasa podrían ser sulfidrilpeptidasas o serin-proteasas. (© Elsevier, Paris)

***Citrus* / germinación / semilla / actividad enzimatica**