Kunitz-type proteinase inhibitors from intact and *Phytophthora*-infected potato tubers

Tatyana A. Valueva*, Tatyana A. Revina, Galina V. Kladnitskaya, Vladimir V. Mosolov

A.N. Bakh Institute of Biochemistry, Russian Academy of Sciences, Leninsky prospekt 33, 117071 Moscow, Russia

Received 12 February 1998; revised version received 3 March 1998

Abstract Three protein proteolytic enzyme inhibitors with molecular masses 21, 22, and 23 kDa have been isolated from intact potato tubers (*Solanum tuberosum* L. cv. Istrinskii). The 21 and 22 kDa proteins denoted as PSPI-21 and PSPI-22, respectively, are serine proteinase inhibitors with different specificity. The 23 kDa protein denoted as PCPI-23 is an inhibitor of plant cysteine proteinases. The PSPI-21 molecule consists of two disulfide-linked polypeptide chains with molecular masses of 16.5 kDa and 4.5 kDa. The PSPI-22 and PCPI-23 have one polypeptide chain. Their amino-termini numbered 21–25 amino acid residues have significant homology to other plant inhibitors which are members of the soybean Kunitz inhibitor family. It is found that at least PSPI-21 and PSPI-22 can predominantly accumulate in potato tubers infected with *Phytophthora infestans* zoospores.

Key words: Kunitz-type proteinase inhibitor; Potato tuber; *Phytophthora infestans*

1. Introduction

Proteolytic enzyme inhibitors are found in plants of various systematic groups. They comprise plant proteins which differ sufficiently in properties. According to homology, number and location of disulfide bridges and reactive site position, there are ten structural groups (families) of plant proteinase inhibitors [1]. One of them is a superfamily of the soybean Kunitz trypsin inhibitor (SKTI) [2]. Kunitz-type proteinase inhibitors which members are mostly single chain polypeptides of 20–24 kDa with four cysteines linked in two disulfide bridges, and with one reactive site located in a loop defined by disulfide bridge, have been found mainly in legume and cereal seeds [2–5]. It is only recently that a group of proteins from potato tubers having molecular masses ranging from 20 to 25 kDa has been described. Some of these proteins have been found to be various Kunitz-type proteinase inhibitors such as trypsin and/or chymotrypsin inhibitors [6,7], subtilisin inhibitor [7], cathepsin D inhibitors [8,9], and papain and/or cathepsin L inhibitor [10]. These inhibitors may play a significant role in the natural defense mechanisms of the potato plant against insect and phytopathogen attack [6,7,9,11]. In this paper, we describe three proteinase inhibitors from potato tubers that have differing inhibitory activities but are Kunitz-type inhibitors based on amino-terminal sequence homologies. In addition, the amino-terminal sequences of three chymotrypsin inhibitors accumulated in potato tubers infected by *Phytophthora infestans* are reported and compared with those of the inhibitors from intact tubers.

2. Materials and methods

Mature potato tubers (*Solanum tuberosum* L. cv. Istrinskii) with *Phytophthora* resistance gene R1 used for protein isolation and for infection with *P. infestans* were stored at 4°C for 2 to 4 months in dark prior to use in experiments. A zoospore suspension (5×10^4 conidia/ml) of *P. infestans* Mont. De Bary race 1.3 from a surface of 11-days mycelium grown on an oatmeal agar medium was used for an inoculation of potato tubers. Indirect zoospore germination was stimulated by keeping ones at 3°C for 1 h. Enzymes: bovine trypsin, α-chymotrypsin, human leukocyte elastase (HLE), papain, and substrates: *N*,α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA), *N*-succinyl-L-alanyl-L-alanyl-L-valine-*p*-nitroanilide (Suc-Ala-Ala-Val-pNA), and *N*-succinyl-L-valyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (Suc-Val-Pro-Phe-pNA) were from Sigma (USA); L-cysteine and EDTA were from Serva (Germany); chemicals, and marker kit for SDS-PAGE were purchased from Pharmacia (Sweden).

Protein inhibitors with molecular masses 21, 22, and 23 kDa (PSPI-21, PSPI-22, and PCPI-23) were isolated from intact potato tubers, purified to homogeneity according to the procedures described previously [12,13].

Diffusates of potato tubers infected with *P. infestans* zoospores were prepared as described previously [14]. In order to detect the inhibitors with affinity to chymotrypsin, clear diffusates were applied to a chymotrypsin-Sepharose column (3.0×3.0 cm) at pH 8.0. The bound proteins were eluted with 7 M urea at pH 3.0.

SDS polyacrylamide gel (20%) electrophoresis (SDS-PAGE) in presence and absence of β-mercaptoethanol was carried out as described by Laemmli [15].

The amino acid sequences were determined on Applied Biosystems model 470 A gas-liquid protein sequencer [16]. Phenylthiohydantoins of amino acids were identified in model 120 APTH amino acid analyzer (Applied Biosystems).

Inhibitory activities were determined by measuring the residual activity of the target enzyme after preincubation with inhibitor. The tested proteinases were assayed using suitable chromogenic substrate, and optimal conditions (pH, temperature, salt concentration) [17–20]. Assuming a slot tight binding mechanism for the enzyme-inhibitor interaction [21], K_i values were calculated by means of the Enzfitter program.

Antifungal activity of chymotrypsin inhibitors isolated from *Phytophthora*-infected tuber diffusates by the affinity chromatography on immobilized chymotrypsin has been measured. Solutions (0.02 M phosphate buffer, pH 6.8) with various concentrations of the inhibitors were added to the zoospore suspension (5×10^4 conidia/ml) and incubated at 19°C for 24 h. The fungal hyphaes were measured under a microscope of Laboval model ($\times120$). Their length was expressed as a percentage of control. Control were zoospores incubated without inhibitors.

3. Results

Purification of PSPI-21 yielded a non-reduced protein with molecular mass of 21 kDa (data not shown). The SDS-PAGE showed that the reduced PSPI-21 contains two polypeptides of molecular masses of 16.5 and 4.5 kDa (Fig. 1A, lane 1).

*Corresponding author. Fax: +7 (95) 954-27-32.
E-mail: inbio@glas.apc.org

PII S0014-5793(98)00321-4

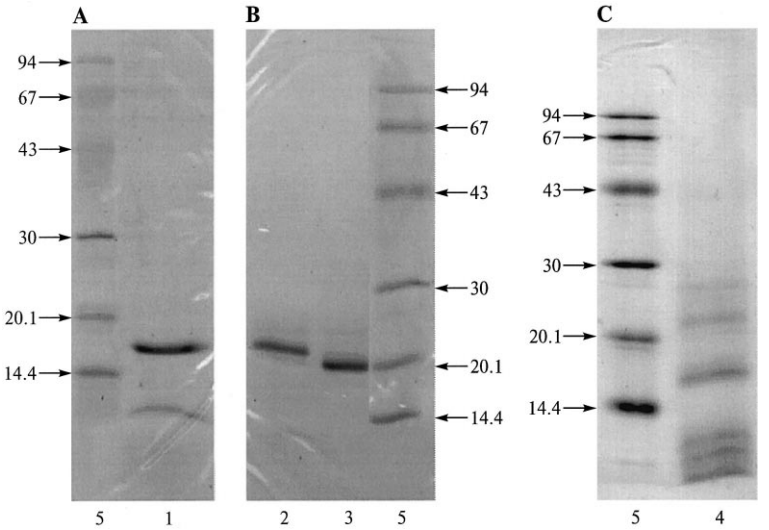

Fig. 1. SDS-PAGE of the proteins purified from intact (A, B) and *Phyrophthora*-infected (C) potato tubers. Lane 1, the PSPI-21; lane 2, the PCPI-23; lane 3, the PSPI-23; lane 4, the proteins purified by affinity chromatography on chymotrypsin-Sepharose from *Phytophthora*-infected tuber diffusates; lane 5, Pharmacia low molecular mass standards (molecular mass $\times 10^{-3}$).

These data demonstrate that a molecule of the homogeneous PSPI-21 consists of two polypeptide chains linked by disulfide bond. The large polypeptide chain was named as A-chain, and the small one was named as B-chain.

In SDS-PAGE, both reduced PSPI-22 and reduced PCPI-23 contained a single polypeptide of molecular mass of 22 and 23 kDa, respectively (Fig. 1B, lanes 3 and 2). These data indicate that the purified proteins are homogeneous and their molecules have one polypeptide chain.

The PSPI-21, the PSPI-22, and the PCPI-23 were subjected to N-terminal amino acid sequence analysis. The native, active PSPI-21 gave two sequences up to 25 residues for A-chain: LPSDATPVLDVTGKELDSRLSYRII..., and up to 10 residues for B-chain: SSDDQFCLKV.... The native, active PSPI-22 gave a single sequence up to 21 residues: FVLPFDVLDSGRDLDRGWPYA.... A single N-terminal sequence of the native PCPI-23 up to 22 residues was PVLQVVRDIHGDILTPDSRYII.... Three sequences had significant homology (approximately 33% identical residues) to each other.

K_i values determined for the PSPI-21, PSPI-22, and PCPI-23 inhibition of some target proteinases are represented in Table 1. The PSPI-21 tightly inhibits HLE, whereas its interaction with trypsin and chymotrypsin is substantially weaker. This protein is not active toward papain, plant cysteine proteinase. The PSPI-22 is a potent inhibitor of trypsin. It exhibited weak inhibition of chymotrypsin and none against papain. In contrast to both PSPI-21 and PSPI-22, the PCPI-23 tightly inhibits only activity of papain, but does not interact with the serine proteinases tested.

In our previous work [14] we have shown that protein chymotrypsin inhibitors accumulate in droplet diffusates of potato tubers in response to *P. infestans* infections. We investigated the chymotrypsin inhibitors which are accumulated in tuber diffusates after 72 h inoculation by *P. infestans* zoospores. In Fig. 1C, the results obtained by SDS-PAGE of the reduced inhibitors purified by affinity chromatography on chymotrypsin-Sepharose show a number of proteins with different molecular masses. The proteins with molecular masses 22.0, 16.5, 8.0, 4.5, and 3.0 kDa are most prevalent, and three of them (with molecular masses 22, 16.5 and 4.5 kDa) are similar to the proteins from intact potato tubers.

To determine N-terminal amino acid sequences, the proteins separated by SDS-PAGE were transferred onto polyvinylidene difluoride membrane by blotting. The protein bands, corresponding to each protein were cut out, washed in water, and dried prior to sequencing, which revealed the N-terminal sequences for four of them. In case of the protein fraction with molecular mass 3 kDa, the N-terminal sequence could not be identified. This fraction was a mixture of several low molecular mass peptides. A presence of such potato tuber peptides which are products of the posttranslational modification of some proteinase inhibitors have been found early [22]. The first ten residues of the protein with molecular mass 22 kDa found in *Phytophthora*-infected potato tuber diffusates were FVLPFDVLDS.... This sequence appeared to

Table 1
Dissociation constants of complexes between potato inhibitors and target proteinases

Proteinase (nM); (Substrate, mM)	K_i, nM		
	PSPI-21	PSPI-22	PCPI-23
Trypsin (4.71); (BAPNA, 0.17)	1.52 (±0.3)[a]	0.58 (±0.6)	n.i.[b]
Chymotrypsin (14.0); (Suc-Val-Pro-Phe-pNA, 0.03)	1.81 (±0.2)	24.90 (±0.9)	n.i.
HLE (4.50); (Suc-Ala-Ala-Val-pNA, 0.10)	0.8 (±0.02)	n.i.	n.i.
Papain (250.0); (BAPNA, 1.50)	n.i.	n.i.	0.32 (±0.03)

[a]Standard deviation ($n = 3$).
[b]N.i., no inhibition.
K_i values were determined from inhibition of enzymatic activities at equilibrium using different concentrations of inhibitors.

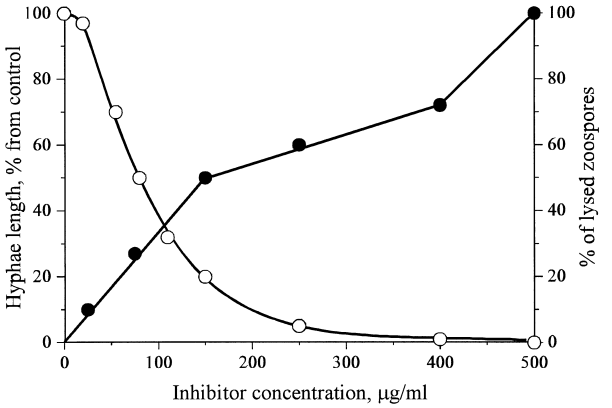

Fig. 2. Length of hyphae (○) and number of lysed zoospores (●) of *P. infestans* as a function of the concentration of chymotrypsin inhibitors purified by affinity chromatography on chymotrypsin-Sepharose from fungus-infected potato tubers. Means of three independent experiments with standard errors from 2 to 10% are presented.

be identical to that of the PSPI-22 from intact tubers which is characterized in this work. The first ten residues of the protein with molecular mass 16.5 kDa were LPSDATPVLD..., and they were identical to those of A-chain of PSPI-21 from intact tubers. The first seven residues of the protein with molecular mass 4.5 kDa were SSDDQFC..., and they were identical to those of B-chain of the PSPI-21. The amino-terminal sequence of the protein with molecular mass 8 kDa (EFDCDGKINW...) was almost identical to that of the potato proteinase inhibitor I promoter A (PI-1A) [23]. These data allow us to conclude that the accumulation of the PSPI-21, the PSPI-22, and PI-1A are induced in potato tubers in response to *Phytophthora* infection. It will be noted that PI-1A is absent in intact tubers of the Istrinskii cultivar [12,14]. The accumulation of this inhibitor is induced in response to the pathogen attack.

The effect of chymotrypsin inhibitors isolated from infected tuber diffusates by affinity chromatography on *P. infestans* growth and development has been studied. The inhibitors significantly inhibit mycelial growth of fungus (Fig. 2). The concentration of the inhibitors that decreases mycelial growth by half is 80 μg/ml. Higher inhibitor concentration (500 mg/ml) completely inhibits fungal growth. The ED_{50}, i.e. a dose at which 50% of zoospores were affected by the potato chymotrypsin inhibitors, is 150 μg/ml. No intact zoospores were observed in the presence of 500 μg/ml inhibitors (Fig. 2); all of them were lysed. Thus, the chymotrypsin inhibitors accumulated in *Phytophthora*-infected potato tuber diffusates are toxic to this microorganism; they inhibited both hyphal growth and zoospore germination.

4. Discussion

We have characterized three inhibitors from intact potato tubers that are distinct from each other in N-terminal amino

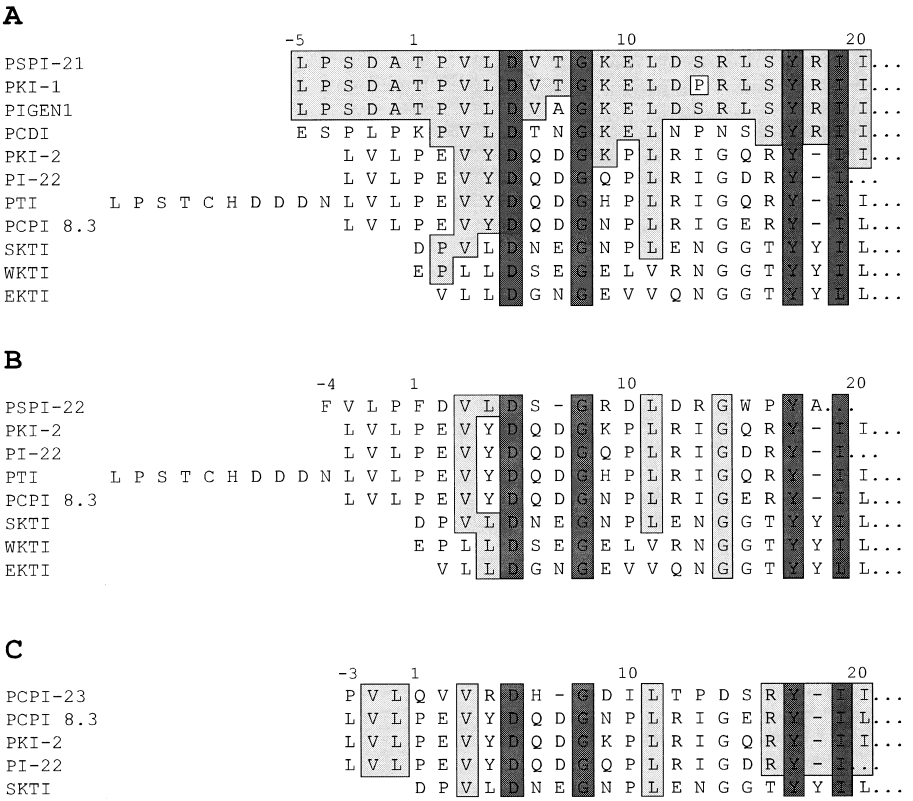

Fig. 3. Comparison of N-terminal sequences of the PSPI-21 (A), the PSPI-22 (B), and the PCPI-23 (C) with other Kunitz-type plant proteinase inhibitors. Identical residues are boxed. Conservative residues are darked boxed. Numbering is made according to the sequence of SKTI [2]. Key: potato inhibitors: PKI-1, the trypsin inhibitor [6]; PKI-2, the subtilisin inhibitor [6]; PCDI and PIGEN1, the cathepsin D inhibitors [8,9]; PTI, the trypsin inhibitor [25]; PI-22, the 22 kDa inhibitor of serine proteinases [7]; PCPI 8.3, the cysteine proteinase inhibitor with pI value of 8.3 [10]; legume inhibitors: SKTI [2]; WKTI, the trypsin inhibitor from winged beans (*Psophocarpus tetragonolobus* (L). DC) [3]; EKTI, the trypsin inhibitor from *Erythrina latissima* seeds [4].

acid sequences and inhibitory activities. Two of them, PSPI-21 and PSPI-22, are serine proteinase inhibitors, but PCPI-23 is a cysteine proteinase inhibitor. Molecules of two inhibitors, PSPI-22 and PCPI-23, have a single polypeptide chain, but PSPI-21 consists of two disulfide-linked polypeptide chains; A-chain is large (16.5 kDa) and B-chain is small (4.5 kDa). All three proteins have homologous sites at the amino-termini. These sequences used to search through the Protein Identification Resource, released 33 protein databases of protein sequences [24]. All three sequences had significant homology to several Kunitz-type inhibitors from a variety of plant sources. Fig. 3A, B, and C show comparisons of the N-terminal sequences of the PSPI-21, the PSPI-22 and the PCPI-23 with other members of Kunitz-type inhibitor family [2–10,25]. All three potato proteins show conservation of residues: Asp-5, Gly-8, Tyr-17, and Ile-19, that are characteristic of Kunitz-type inhibitors.

Although these inhibitors are clearly members of Kunitz-type inhibitor family, they bear more similarity to some potato inhibitors that are members of this family than to each other. The PSPI-21 is homologous to both potato trypsin inhibitor (PKI-1) [6] and potato cathepsin D inhibitor (PIGEN1) [9], being identical in residues −5 to 20, excluding 7 and 13 residues differ; Tyr-7→Ala-7 (PIGEN1) and Ser-13→Pro-13 (PKI-1). However, PSPI-21 differs from both PKI-1 and PIGEN1. First of all, the PSPI-21 is double chain protein. Besides, these inhibitors differ in a specificity of the action on enzymes. As opposed to PKI-1 [6], PSPI-21 is a potent inhibitor of HLE, but a good inhibitor of trypsin, and of chymotrypsin. PIGEN1 is inhibitor of cathepsin D, aspartic proteinase [9]. Our previous work, however, established that PSPI-21 does not inhibit aspartic proteinases (cathepsin D and pepsin) [13]. Amino-terminal sequences of the PSPI-22 and the PCPI-23 indicate a high homology (about 30% and 45%, respectively) with those of potato subtilisin inhibitor (PKI-2) [6], potato trypsin inhibitors (PI-22 and PTI) [7,25], and potato cysteine proteinase inhibitor (PCPI 8.3) [10]. However, in contrast to PKI-2 and PCPI 8.3 which are potent inhibitors of subtilisin and of cathepsin L, respectively, the PSPI-22 did not demonstrate activity towards subtilisin and cysteine proteinases [13]. This protein is a potent inhibitor of trypsin. In turn, the PCPI-23 is a potent inhibitor of plant cysteine proteinase, papain, being different from PKI-1, PI-22, and PTI. A significant high degree of amino acid similarity between the analyzed protein homologues suggests that all of them are members of one multigene family of potato Kunitz-type proteinase inhibitors (PKPIs).

As we are aware, the effect of phytopathogen attack on proteinase inhibitor accumulation in potato tubers has not been studied yet. There is an evidence that the level of potato inhibitor II mRNA increases in detached tubers in response to wounding [26]. However, when wounded tubers are attached to the plant, the increase in the mRNA content was less obvious [27]. We have indicated that the infection by *P. infesnans* zoospores can induce the accumulation of chymotrypsin inhibitors in attached potato tubers too. This work showed that the PSPI-21, the PSPI-22, and the PI-1A were prevalently accumulated in response to the fungus infection. Because these inhibitors suppress both zoospore germination and growth of the *P. infestans* hyphae, they are block fungal development. These data allow us to suggest that proteinase inhibitor genes may be regulated both developmentally and environmentally in attached potato tubers.

Acknowledgements: This work was supported by Grant 96-04-48097 from Russian Foundation for Basic Research.

References

[1] Garcia-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomez, L., Royo, J. and Carbonero, P. (1987) in: B.J. Miflin (Ed.), Oxford Surveys of Plant Molecular and Cell Biology Vol. 4, Oxford University Press, Oxford, pp. 275–334.
[2] Kim, S.H., Hara, S., Hasa, S., Ikenaka, T., Toda, H., Kitamura, K. and Kaizuma, N. (1985) J. Biochem. 98, 435–448.
[3] Yamamoto, M., Yara, S. and Ikenaka, T. (1983) J. Biochem. 94, 849–862.
[4] Joubert, F.J., Heussen, C. and Bowdle, B.D. (1985) J. Biol. Chem. 260, 12948–12953.
[5] Svendsen, I., Heijgaard, J. and Chavan, J.K. (1984) Carlsberg Res. Commun. 51, 43–50.
[6] Walsh, T.A. and Twitchell, W.P. (1991) Plant Physiol. 97, 15–18.
[7] Suh, S.-G., Stiekema, W.J. and Hannapel, D.J. (1991) Planta 184, 423–430.
[8] Mares, M., Meloun, B., Pavlik, M., Kostka, V. and Baudys, M. (1989) FEBS Lett. 251, 94–98.
[9] Strukelj, B., Pungercar, J., Mesko, P., Barlic-Magana, D., Gubensek, F., Kregar, I. and Turk, V. (1992) Biol. Chem. Hoppe-Seyler 373, 477–482.
[10] Krizaj, M., Drobnic-Kosorok, J., Brzin, J., Jerala, R. and Turk, V. (1993) FEBS Lett. 333, 15–20.
[11] Ishikawa, A., Ohta, S., Matsuoka, K., Hattori, T. and Nakamura, K. (1994) Plant Cell Physiol. 35, 303–312.
[12] Revina, T.A., Valueva, T.A., Ermolova, N.V., Kladnitskaya, G.V. and Mosolov, V.V. (1995) Biochem. (Mosc.) 60, 1411–1416.
[13] Valueva, T.A., Revina, T.A. and Mosolov, V.V. (1997) Biochem. (Mosc.) 62, 1367–1374.
[14] Kladnitskaya, G.V., Valueva, T.A., Ermolova, N.V., Il'inskaya, L.I., Gerasimova, N.G. and Mosolov, V.V. (1996) Russ. J. Plant Physiol. 43, 611–615.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Henschen, A. (1986) in: B. Wittmann-Liebold, J. Salnikow and V.A. Erdmann (Eds.), Advanced Methods in Protein Microsequence Analysis, Springer, Berlin, pp. 244–255.
[17] Powers, J.C., Tanaka, T., Harper, J.W., Minematsu, Y., Barker, L., Lincoln, D., Grumley, K.V., Fraki, J.E., Schechter, N.M., Lazarus, G.G., Nakajima, K., Nakashino, K., Neurath, H. and Woodbury, R.G. (1985) Biochemistry 24, 2048–2058.
[18] Wenzel, H.R., Engelbrecht, S., Reich, H., Mondry, W. and Tschesche, H. (1980) Hoppe-Seyler Z. Physiol. Chem. 361, 1413–1416.
[19] Kakade, M.L., Simons, N. and Liener, I.L. (1969) Cereal Chem. 46, 518–526.
[20] Barrett, A.J., Kembhavi, A.A., Brown, M.A., Kirschke, H., Knight, C.G., Tamai, M. and Hamada, K. (1982) Biochem. J. 211, 189–195.
[21] Knight, C.G. (1986) in: A.J. Barrett and G. Salvesen (Eds.), Proteinase Inhibitors, Elsevier, Amsterdam, pp. 23–51.
[22] McManus, M.T., Laing, W.A., Cristeller, L.T. and White, W.R. (1994) Plant Physiol. 106, 771–777.
[23] Richardson, M. and Cossins, L. (1975) FEBS Lett. 45, 11–13.
[24] Dayhoff, M.O., Barker, W.C. and Hunt, L.T. (1983) Methods Enzymol. 91, 524–545.
[25] Yamagishi, K., Misumori, C. and Kikuta, Y. (1991) Plant Mol. Biol. 17, 287–288.
[26] Sanchez-Serano, J., Schmidt, R., Schell, J. and Willmitzer, L. (1986) Mol. Gen. 203, 15–20.
[27] Pena-Cortes, H., Sanchez-Serano, J., Rocha-Sosa, M. and Willmitzer, L. (1988) Planta 174, 84–89.