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ACTIVATORS AND INHIBITORS OF PROTEASE FROM COTYLEDONS OF *CUCURBITA* SEEDLINGS.

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ABSTRACT

Protease was isolated from cotyledons of 5-day old marrow (*Cucurbita pepo* L.) seedlings. The enzyme was induced by kinetin or benzyladenine (BA) at 50, 100 and 150 µmol. Protease was also induced by homobrassinolide (HBL) after treatment with 0.2, 0.4, 0.6, 0.8 or 1.0 µmol. Polyamines such as putrescine, spermine, cadaverine, spermidine and mixture of polyamines induced protease activity. Ca^{2+}, K^{+} and Mg^{2+} activated protease activity when tested at either 5 or 10 mM. On the other hand, Co^{2+}, Zn^{2+}, Al^{3+}, Cu^{2+} and Hg^{2+} inhibited the enzyme activity. AMP, ADP and ATP induced protease activity. Surfactants such as Triton X-100, Tween 60 and Tween 80 increased protease activity when added in the range of 0.2-1.2 % (v/v). Treatment with various concentrations of $CdCl_2$ and $PbCl_2$ resulted in inhibition of protease activity and $PbCl_2$ was the stronger inhibitor. EDTA as chelating agent inhibited protease activity.

INTRODUCTION

Proteases are biocatalysts which have been in use for many centuries, at first in the dairy industry as milk-clotting agents and is generally done by using the rennin enzyme for the manufacture of cheese. This protease enzyme is obtained from the stomach of calves (Cavalcanti *et al.,* 2004).

Proteases have critical functions in many normal and aberrant biochemical processes that occur at the cellular and organismal levels (Puente & López-Otin, 2004).

Cytokinins are adenine-derivative molecules with diverse active forms. Zeatin is an important cytokinin found in higher plants (Dello-Ioio *et al.*, 2008).

Cytokinins play positive roles in germination, root and shoot development, and nodulation, and appose leaf senescence and pathogen invasion (Sakakibara, 2006; To & Kiber, 2008; Acharya & Assmann, 2009).

Cytokinins are plant growth regulators that play a major role in cell division and cell differentiation. They can stimulate shoot differentiation, bud formation, the growth of lateral buds, leaf expansion, and chlorophyll synthesis. They can also enhance resistance to salinity (Dodd, 2003).

Brassinosteroids were found to be involved in increasing resistance to chilling stress in brome grass (Wilen *et al*., 1995) and rice (Wang & Zang, 1993). In *Eucalyptus camaldulensis*, treatment of seeds with 24-epibrassinolide resulted in an increase in seed germination under saline conditions (Vardhini & Rao, 2002).

28-homobrassinolide (HBL) was found to alleviate the salinity-induced inhibition of germination and seedling growth in rice (Anuradha & Rao, 2001). Also, seed treatment with very dilute solutions of brassinosteroids considerably improved the growth of rice plants in saline media (Kamuro & Takatsuto, 1999).

Polyamines are a class of growth regulating substances and it has been reported to be involved in several aspects of plant development (Liu *et al.,* 1997).

Like phytohormones, they are present in plants and display biological activity in different processes such as plant growth, development, and stress mitigation. Thus, they are considered multifunctional regulators of plant physiological processes (Kuznetsov *et al*., 2006).

Polyamines were reported to play a role related to the avoidance of lipid peroxidation reactions, preventing the formation of oxidative damage (Tiburcio *et al.,* 1993). It was reported that polyamines behave as antioxidants in the protective mechanisms (Tiburcio *et al.,* 1997).

Polyamines have been also suggested to function as metal chelators and good candidates in protecting plant cell against metal-induced oxidative damage due to their high affinity for biological membranes and because are easily induced in response to stress conditions (Stoeva *et al*., 2005).

Plant metabolism may be affected by cadmium (Cd^{2+}) in different ways. It has been greatly described that cadmium strongly altered plant metabolism, but the intensity of its toxic effects depends both on the plant species used, and on the way and time that plants are cultivated (Boussama *et al.,* 1999; Di Cagno *et al.,* 1999; Romero-Puertas *et al.,* 2002; Hsu & Kao, 2003).

Pb^{2+} inhibits metabolic processes such as nitrogen assimilation, photosynthesis, respiration, water uptake and transcription (Boussama *et al.,* 1999).

The present work aimed to investigate the activators and inhibitors of protease from marrow cotyledons.

MATERIALS AND METHODS

Seed germination and growth conditions

Seeds of *Cucurbita* were germinated according to El-Shora (2001) as follows:

1- The seeds of different plants were surface sterilized in 10 % sodium hypochlorite for 10 min, soaked in running tap-water for 24 h, and then germinated between paper towels, moistened with distilled water in sterilized plastic trays. The trays were covered and incubated in the dark at 25 °C for 48 h.

2- The germinated seeds with well-grown roots were then supported on plastic bowls containing 0.2 mM $CaCl_2$ solution. $CaCl_2$ solution was continuous and vigorously aerated. Seeds were grown for further 48 h in light at 25 °C. The cotyledons and roots of 5-days old plants were excised with a razor blade and kept on ice to be used for enzyme extraction immediately.

Crude enzyme extraction

Cotyledons (100 gm) of each treatment as well as control samples were suspended in 5 ml of pre-chilled 20 mM phosphate buffer (pH 7.0) and the mixture was blended in warren blender and stirred, the resulting homogenate was centrifuged at 10000 g for 10 min. The resulting

supernatant constitutes the crude enzyme extract which was used for subsequent analysis.

Assay for protease activity

The proteolytic activity was determined using casein as the substrate (Fujiwara *et al.,* 1993). One unit is defined as µmol products per min at 25°C

Treatment with kinetin and 6-benzyladenine (6-BA)

The cotyledons of 5-day old seedlings of marrow were incubated with 20 ml of distilled water (control) or with kinetin or 6-BA at either 50, 100 and 150 µmol for 72 h at 25 °C followed by determination of the protease activity.

Treatment with 28-homobrassinoloide (HBL)

The cotyledons of 5-day old seedlings of marrow were incubated with 20 ml of distilled water (control) or with various concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 µmol) of HBL for 72 h at 25 °C followed by determination of the protease activity.

Effect of polyamines

Cotyledons of 5-day old seedlings were treated by 1 mM of putrescine, spermine, cadaverine, spermidine or a mixture of the polyamines for 72 h. Control samples to which no polyamines was added were carried out.

Effect of metal ions

The tested ions are examined as metal chloride, $CaCl_2$, KCl, $MgCl_2$, $CoCl_2$, $ZnCl_2$, $AlCl_3$, $CuCl_2$ and $HgCl_2$. they are tested at either 5 or 10 mM in the enzyme. Control samples to which no metal chloride was added were carried out.

Effect of adenosine compounds

Each of ATP, ADP and AMP are examined at various concentrations (0.2, 0.4, 0.6, 0.8 and 1.0) mM in the assay medium. Control samples were carried out.

Effect of surfactants

The effects of the surfactants as Triton X-100, Tween 60 and Tween 80 on the protease activity was studied at various concentrations 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 % by incubating the isolated enzyme from marrow cotyledons in the presence of the surfactant for a period of 1 h, and the residual activity was determined.

Effect of $CdCl_2$ and $PbCl_2$

$CdCl_2$ and $PbCl_2$ were tested by incubating the cotyledons of 5-day old seedlings of marrow with various concentrations 20, 40, 60, 80 and 100 µmol of each compound for 72 h. Control samples were carried out.

Effect of EDTA

EDTA as chelating agent was tested at various concentrations 10, 20, 30, 40 and 50 µM in the assay medium of protease. Control samples were carried out.

Statiscal analysis

All values are mean values ± SE.

RESULT AND DISCUSSION

Treatment of marrow cotyledons with kinetin and 6-BA resulted in induction of protease activity (Fig. 1). Kinetin treatment increased the activities of other plant enzymes such as carbonic anhydrase (Hayat *et al*., 2001b).

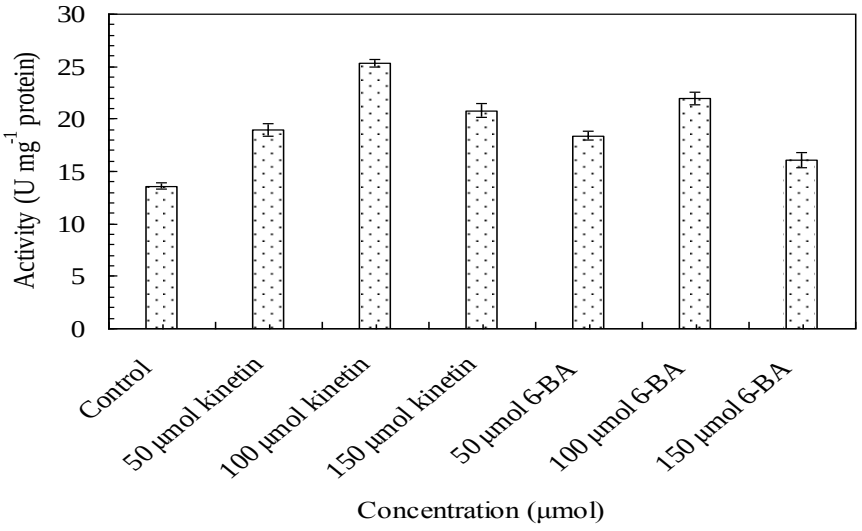

Fig. 1: Effect of kinetin and 6-benzyladenin on protease activity.

Kinetin treatment increased the activity of other plant enzymes such as carbonic anhydrase (Hayat *el al.,* 2001b). The authors suggested that cytokinins can increase the enzyme activity through acting either at the level of transcription and/or the stabilization of the transcripts as they increased the level of carbonic anhydrase-mRNA.

Application of 6-BA to marrow cotyledons resulted in an increase in the activities of protease. 6-BA increased the activities of other plant enzymes such as glutamate oxaloacetic acid transaminase (Abdel-Gaffar, 1999), phytase and acid phosphatase (Mohammed, 2003) and RNA polymerase (Ananiev *et al*., 2003).

Increased synthesis of specific enzymes by cytokinins is also reported for plant enzymes such as nitrate reductase (Lu *et al.,* 1990), ribulose 1-5-bisphosphate carboxylase (Ohya & Suzuki, 1991), glutamyl-tRNA reductase and glutamate-1-semialdehyde aminotransferase (Yaronskaya *et al.,* 2006), RNA polymerase (Ananiev *et al.,* 1987), phosphoenolpyruvate carboxylase, carbonic anhydrase (Sugiharto *et al.,* 1992), glutamate synthase (El-Shora, 2001) and acid phosphatase (El-Shora & Metwally, 2009).

In addition, it has been reported that cytokinins strongly increased the level of mRNA encoding NADPH-protochlorophyllide oxidoreductases and the content of this enzyme in cucumber and lupine cotyledons (Kuroda *et al.,* 2001). It seems reasonable to conclude that cytokinin-induced increase in protease activity could be due to an increase in the amount of the enzyme protein.

Treatment of cotyledons with different concentrations tested of HBL resulted in continous increase of protease activity (Fig. 2). However, the exact mode of action of HBL is not known but Hayat *el al.* (2001a) are of the opinion that HBL could act on the enzymes in a way similar to that of kinetin. HBL have also earlier been claimed to be the elevator for the activities of other plant enzymes such as nitrate reductase (Hayat *el al.,* 2001b; Hayat & Ahmed, 2003) and L-asparaginase (El-Naqeeb, 2009).

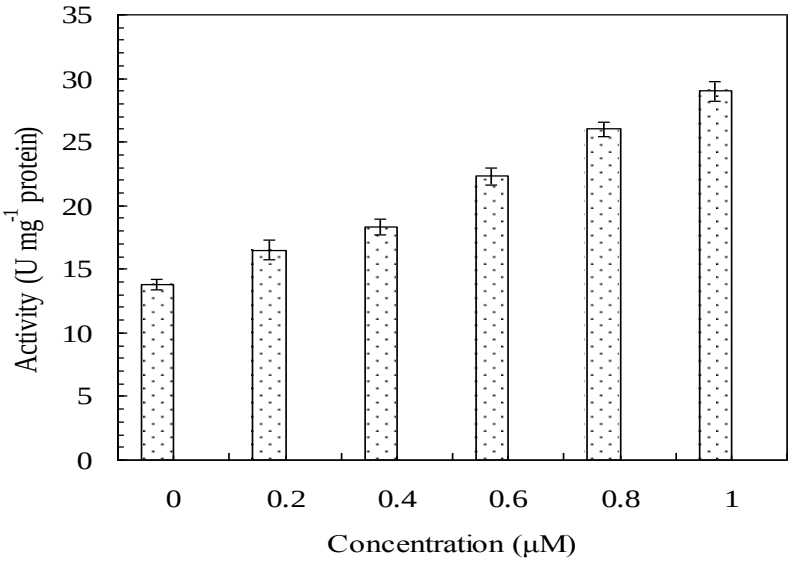

Fig. 2: Effect of homobrassinolide (HBL) on protease activity.

Furthermore, HBL as a steroidal phytohormone expressed a wide range of physiological responses in plants such as synthesis of nucleic acid and proteins (Yu *et al.,* 2004).

Protease of marrow cotyledons was activated by the various tested polyamines and spermine and cadaverine were the best activators (Fig. 3).

Polyamines stimulated other enzymes such as AMP deaminase (Yoshino & Murakami, 1982), protein kinase about two fold (Meksuriyen *et al*., 1998) and acid phosphatase from potato tubers (Tanemura & Yoshino, 2006). Activating effect of polyamines on various enzymes has been explained by affecting the binding of metal (Lahti *et al*., 1989) required for catalysis (Yoshino & Murakami, 1982).

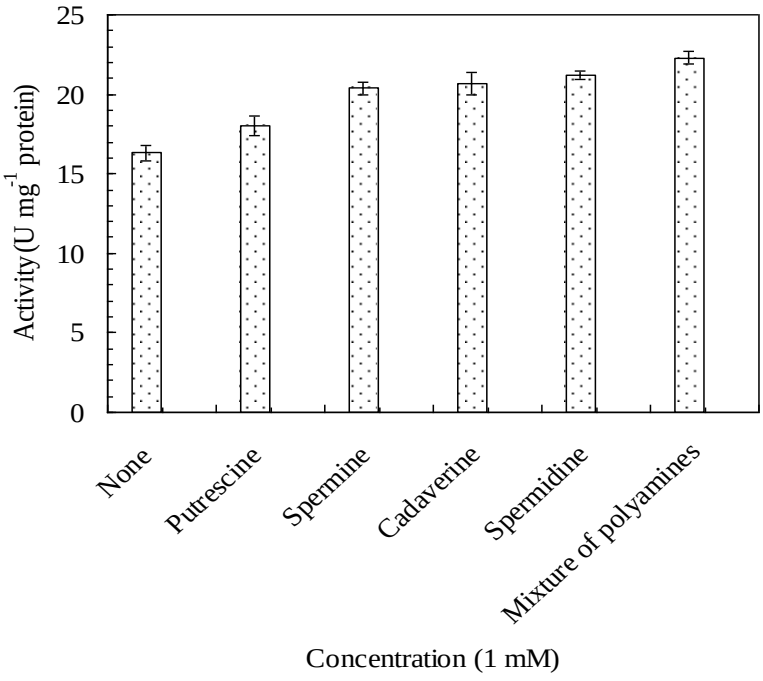

Fig. 3: Effect of various polyamines on protease activity.

Cu^{2+} and Hg^{2+} on protease activites (Fig. 4). It was found that only Ca^{2+}, K^{+} and Mg^{2+} induced protease activity. Ca^{2+} was the best divalent cation.

Treatment of protease with $CaCl_2$ resulted in the increase of its activity. A reason for an increase in activity of enzyme in the presence of calcium may be due to stabilization of enzyme in its active conformation rather than it being involved in the catalytic reaction. It probably acts as a salt or ion bridge *via* a cluster of carboxylic groups as has been suggested for subtilisins and thereby maintains the rigid conformation of the enzyme molecule (Strongin *et al.,* 1978).

On studying the effect of adenosine compounds on protease activity in marrow cotyledons (Fig. 5) it was found that there was corresponding increase in protease activity with increasing the concentrations of AMP, ADP and ATP. ATP was the best adenosine compound to activate protease.

The effect of the surfactants on the protease activity was studied. Among the surfactants used in the present study, Triton X-100, Tween 60 and Tween 80. The increase of enzyme activity was most remarkable with Triton X-100 up to 1.2 % (v/v) (Fig. 6).

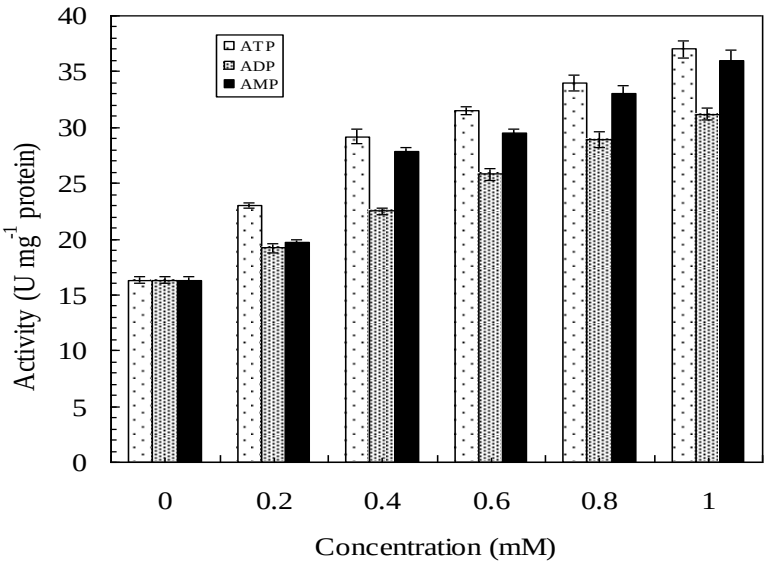

Fig. 5: Effect of adenosine compounds on protease activity.

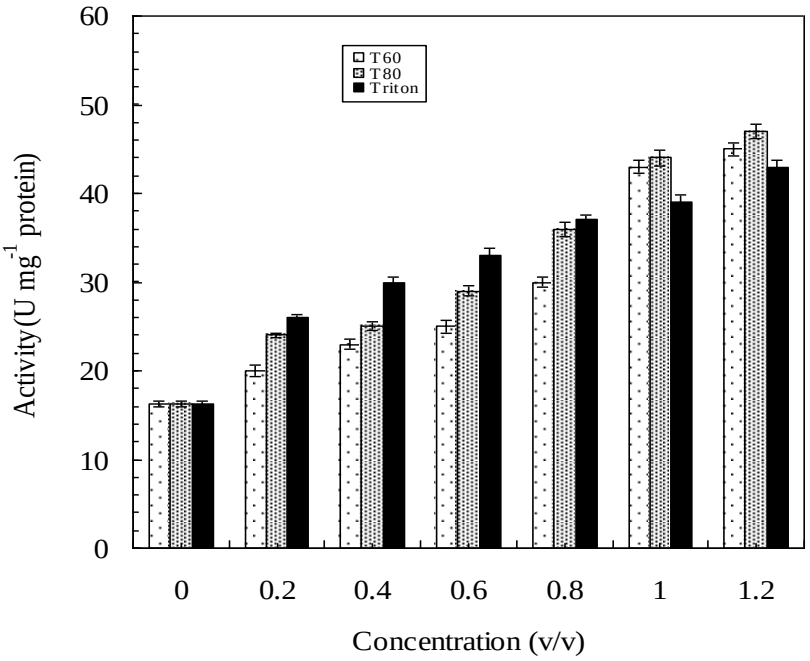

Fig. 6: Effect of surfactants on protease activity.

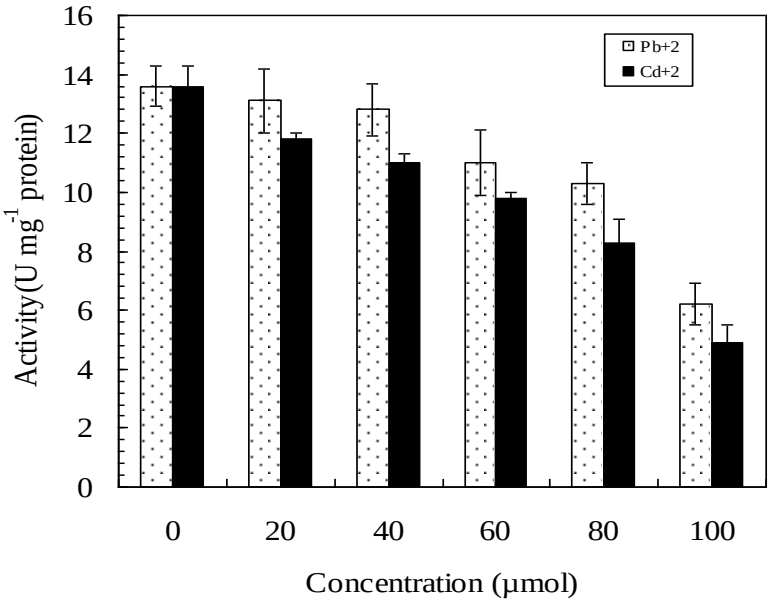

Fig. 7: Effect of $CdCl_2$ and $PbCl_2$ on protease.

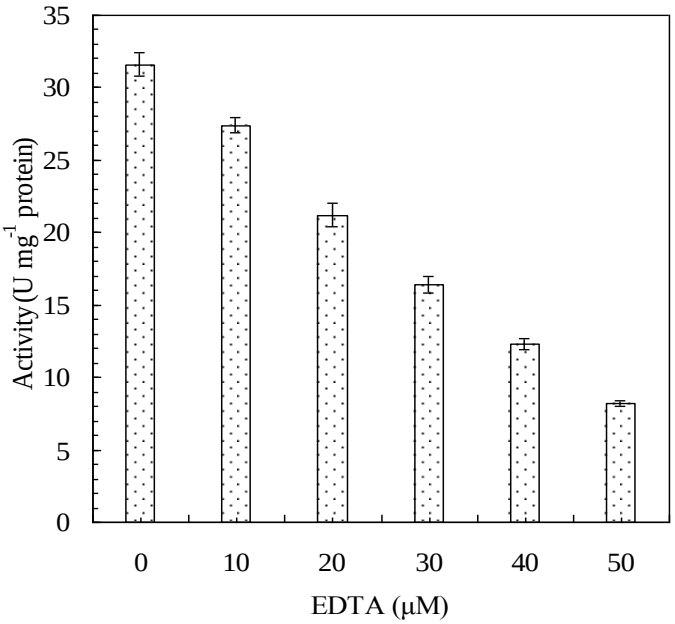

Fig. 8: Effect of EDTA on protease enzyme.

The above results are in agreement with those reported for malic enzyme (Guagliardi *et al.,* 1989) and those for ATPase which is enhanced by anionic surfactants (Norling, 1986).

Treatment of marrow cotyledons with $CdCl_2$ and $PbCl_2$ at various concentrations (Fig. 7) resulted in reduction of protease activity. Pb^{2+} was the stronger inhibitor for protease at all the tested concentrations.

The inhibition of the activities of many enzymes with Cd^{2+} and Pb^{2+} has been attributed to the interaction of those metals with functional –SH group present on enzymes (Shah & Dubey, 1995).

An inhibitory effect of EDTA on protease activity was observed (Fig. 8). This effect of EDTA on the enzyme may be a result of Ca^{2+} chelation, which supports hypothesis that the enzyme requires Ca^{2+} for its active conformation.

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المنشطات والمثبطات لانزيم البروتييز من فلقات بادرات القرع

حامد محمد الشورى و هاله سعد طه
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تم فصل انزيم البروتييز من فلقات نبات القرع الناميه لمدة خمسة أيام. أوضحت النتائج أن الكينيتين وأدينين البنزيل قد حفزا النشاط الانزيمى عند استخدامهما بالتركيزات الثلاثه ٥٠, ١٠٠, ١٥٠ ميكرومول. كذلك بينت النتائج أن مركب البرازينوليد عند التركيزات المختلفة ٠,٢, ٠,٤, ٠,٦, ٠,٨, ١,٠ أدى الى زيادة النشاط الانزيمى فى الفلقات. كما أدت معاملة الفلقات بالمركبات عديدة الأمين مثل البتريسين والاسبرمين والكاداڤيرين والاسبرميدين وكذلك خليط من هذه المركبات الى استحثاث النشاط الانزيمى للبروتييز عند استخدام هذه المركبات بتركيزات ٥٠, ١٠٠ ميكرومول. أظهرت النتائج أنه تم تنشيط الانزيم فى وجود الكالسيوم والبوتاسيوم والماغنيسيوم عند استخدامهم بتركيزات ٥, ١٠ ملللى مول فى وسط التفاعل. بينما أدت أيونات الكوبلت والزنك والألومنيوم والنحاس والزئبق الى تثبيط النشاط الانزيمى عند استخدامهم بنفس التركيزات ٥, ١٠ ملللى مول. أدت معاملة الانزيم بكل منATP, ADP, AMP الى زيادة النشاط الانزيمى عند اضافتهم بالتركيزات ٠,٢, ٠,٤, ٠,٦, ٠,٨, ١,٠ ملللى مول فى وسط التفاعل. كذلك أدت اضافة المركبات ترايتون والتوين ٦٠ والتوين ٨٠ الى زيادة النشاط الانزيمى عند استخدامهم بالتركيزات ٠,٢, ٠,٤, ٠,٦, ٠,٨, ١,٠, ١,٢ % (v/v) وأظهرت النتائج أن معاملة الفلقات بكلوريد الكادميوم وكلوريد الرصاص بتركيزات مختلفة ٢٠, ٤٠, ٦٠, ٨٠, ١٠٠ ميكرومول الى تثبيط النشاط الانزيمى وكان كلوريد الرصاص أكثر تثبيطا. أدت اضافة الايثيلين داى أمين تيترا أسيتات الى وسط التفاعل كمادة مخلبية الى تثبيط النشاط الانزيمى.

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