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RESEARCH ARTICLE

Isolation and detection of Ɛ-(ɣ-glutamyl) lysine isodipeptide in tissues of broad bean (*Vicia faba*var Aquadulce)seeds at different stages after development

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Abstract

Transglutaminase 2 (TG-ase or TG 2) an ubiquitous enzyme which is present in various living cells; animals, plants, algae and bacteria, and reported to perform diverse roles and functions in developmental processes was investigated in tissues of broad beans (*Vicia faba* var Aquadulce) seeds at different stages (large, medium and small sized seeds) after development. Protein was extracted from two different compartments of the seeds (testa and cotyledon) and was examined for the presence of the enzyme and its activity. The study showed the presence of TG 2 both in the testa and cotyledon of the seeds. Also, transglutaminase activity was detected by the presence of Ɛ-(ɣ-glutamyl) lysine isodipeptide. However, the activity was found to be very high in the testa and cotyledon of the small sized seeds of *Vicia faba* and decreases as the seed grow bigger (mature). The decrease in TG 2 activity is an evidence that the enzyme is involve in some developmental processes that take place in the seeds possibly cell differentiation and cell wall formation

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Introduction

The growth and development of living organisms both plants and animals including some microorganisms is controlled by a wide range of environmental and genetic (gene) factors which is referred to as gene-environment interaction (gei). However, there are a number of physiological processes that take place in living organism; among these is the cross-linking of proteins which is essential in the stabilization of tissues and other cellular matrices (Serafini-Fracassini *et al*., 1995).Bertossi *et al*. (1965) reported the effects of polyamine on plant growth particularly in *Helianthus tuberosus* dormant tubers and later was confirmed in several other plants. The molecular mechanism of action of polyamines is only partially known (Serafini-Fracassini and Del Duca, 2008). These amines are present in free and bound form (Serafini-Fracassini and Del Duca, 2008) which are found in series of animal cells and have been confirmed to have diverse functions catalyzed by certain enzyme family known as transglutaminase most time in the presence of calcium ion.

It has been reported that tissue transglutaminase in plants have similar roles with that of animal counterpart (Serafini-Fracasini *et al*., 2009). The few transglutaminases that has being derived from plant are slightly the same with the derived transglutaminases from animal. The characteristics they share with animal derived transglutaminase according to Del Duca and Fracassini, (2005) include; recognition by animal transglutaminase antibodies, ability to produce glutamyl-polyamine derivatives and the fact that their activity depend on the presence of calcium ion. However, Fracassini and Del Duca (2008) stated that the likely role of type 2 transglutaminase is peculiar to the structural modification of certain protein substrates. In chloroplasts however, tissue transglutaminase or TG2 appears to stabilize the photosynthetic complexes which is majorly regulated by light and other environmental factors. Del Duca *et al*. (1995) also suggested their involvement in the modification, construction and organization

of cell wall, while in the cytosol they modify cytoskeletal proteins. Other roles played by plant TG2 are related to biotic and abiotic factors; fertilization, senescence and programmed cell death (PCD) (Serafini-Fracassini and Del Duca, 2008). However, the transglutaminase present in algae has been reported by Del Duca and Serafini-Fracassini, (2005) to be involved in the protection of the photosynthetic apparatus in algae from stress. Nevertheless, it should not be forgotten that most of their (plant tissue transglutaminase) properties are still remain unclear.

Type 2 transglutaminase activities have been reported in higher plants, although the specific biological role of the enzyme has not been clearly defined as reported by Lilley *et al*., (1998).

Studies have also suggested that Ca^{2+} ions stimulate the activity of plant transglutaminase but are not an absolute requirement (Lilley *et al*, 1998). Furthermore, studies of transglutaminase activity and substrates in leaves, tubers and flower buds of some plants for example; the study conducted on Helianthus, showed that this enzyme is widespread and that in the same organ multiple enzyme forms are present (Del Duca and Serafini-Fracassini, 2005)

However, the object of interest to this research project is the enzyme tissue transglutaminase2. This member of transglutaminase family is involved in three major activities in living cells; deamination, polyamine incorporation and protein cross- linking. The focus of this project will be on the protein cross-linking ability of the type 2 transglutaminase which is caused by the presence of Ɛ-(ɣ-glutamyl) lysine isodipeptide.

The precise evidence for the presence of transglutaminase in living cell is the detection of Ɛ-(ɣ-glutamyl) lysine isodipeptide (Folk and Finlayson, 1977). Meanwhile, initial result from the work of Lilley *et al*. (1998) on both monocotyledonous and dicotyledonous plantlets showed that there is a lot of cross-link in both the roots and shoots (leaves) of the plantlets used (wheat and barley) for monocotyledon and (pea and broad bean) for dicotyledonous plant. The results from the work of Lilley *et al*., (1998) on the root and shoot of monocotyledonous and dicotyledonous seedlings prompted this study.

Thus the objective of this study is to examine the seeds of broad bean at different stages after development for the presence of type 2 transglutaminase and its activity.

Materials and Methods

All materials used, except otherwise stated were purchased from Sigma-Aldrich, UK.

Seeds of broad bean (*Vicia faba* var *Aquadulce*) from different stages after development were separated into testa and cotyledon by the use of scalpel. The divisions were put into different red top tubes (15ml or 50ml) depending on their volume. Each red top tube containing either testa or cotyledon was treated with liquid nitrogen and the red top was perforated before introducing them into the freeze dryer for drying which was for (48 hours or more) depending on the volume.

After drying, the samples were homogenized into powder form using pestle and mortal. The resulting powder was kept in the fridge at 4° C till the time of use.

Dialysis tubing was boiled for 5 minutes in 200.0 ml of distilled water containing 0.1g EDTA and 2.0g sodium hydrogen carbonate. The tubing was then rinsed and boiled again in distilled water for another 5 minutes. Tubing was stored at 4°C in a solution of 20% methanol and was taken out from the fridge at each time of use.

5.0g of mature broad bean cotyledon powder was dissolved into 25ml of extraction buffer and was stirred by rod stirrer until it was evenly mixed. The resulting mixture i.e. the homogenate was filtered through two layers of muslin cloth. The protein extract was aliquoted into eppendorf micro tubes (1.5ml). The extract was centrifuge at 13,000g for 20 minutes at 4°C. 14.5 ml of the supernatant was recovered and 1.0 ml was kept in the freezer as the crude protein. The remaining 13.5ml supernatant was precipitated by addition of 10g ammonium sulphate and was stirred for 2 hours on ice. The precipitated protein was collected by centrifugation at 13,000g for 20 minutes at 4°C in the cold room, re-dissolved in 20mM Tris-HCl pH 8.0, containing 1mM 2-mercaptoethanol and dialysed against 1.5 liter of the same buffer at 4°C overnight. Aliquots of dialyzed protein were stored at -20°C in the freezer.

Plant material was homogenized and protein was precipitated from the 80000×g supernatant by the addition of an appropriate volume of 72% (w/v) TCA to give a final concentration of 10% (w/v). The protein was pelleted by centrifugation at 13000×g for 20 minutes at 4°C using a Jouan model A-14 mini bench centrifuge (St. Nazaire, France). The pellets were washed once in 10% (w/v) TCA (10g of solid TCA was dissolved in distilled water to a final volume of 100.0 ml), three times in 50:50 ethanol: diethyl ether and 3 times in diethyl ether (Each washing was followed by centrifugation at 13000 rpm for 3 minutes using a Jouan model A-14 mini bench centrifuge (St. Nazaire, France). The resultant pellets were dried by evaporation on a non heat cycle (Jouan model RC.10.22 centrifugal evaporator, St Nazaire, France) and re-dissolved in 1.0 ml of 100mM ammonium carbonate pH 10.0. A crystal of thymol was added to each pellet to inhibit microbial growth. The pH of each tube was checked and adjusted to pH 10.0 by the addition of sodium hydroxide. 100µg of subtilisin was added to each tube as 10µl of a 10 mg ml^{-1} solution and the tubes were placed in an incubator at 37°C (Analytical Supplies, Derbyshire, U.K). The

samples were filtered before each addition of the proteolytic enzymes in order to get rid of bacteria and other microbes that might have found their way into the tubes. All subsequent proteolytic enzyme incubations were carried out at this temperature (37°C). 50µl of each digestion was re-dissolved in 50.0µl of loading buffer and the samples were then loaded onto the amino acid analyzer for the amino acid analysis and for the detection of the crosslink.
The protein content of crude and dialyzed plant extracts was determined using a modified bicinchoninic acid method (Brown *et al*., 1989). BSA was used as the standard protein in all the studies conducted.

Enzyme assay was used as a measure of transglutaminase activity and was performed according to the method of Slaughter *et al*. (1992) with the modifications of Lilley *et al*. (1998a) and some other modifications. Ninety-six well NUNC Maxisorp assay plates were coated overnight unless otherwise stated with 250.0µl per well of distilled water containing 10 mg ml^{-1} N, N'-dimethylcasein at 4^oC. After discarding unbound protein, plates were washed twice with 150mM PBS-Tween 80 and twice with distilled water. Plates were blocked with 250.0 µl per well of 100mM Tris-HCl pH 8.5 containing 3% (w/v) BSA (0.75 g) (prepared fresh) and shaken for 45 minutes at room temperature. Plates were washed with distilled water.
To each well was added 150.0µl of calcium chloride assay buffer or 150.0 µl of EDTA assay buffer and a 50.0 µl standard (Standard transglutaminase) (Std or ST) or sample (crude, dialysed and purified mature broad bean cotyledon extract). Plates were incubated for 120 minutes (2 hours) at 37°C and then washed as described previously. To each well was added 200.0µl of 100mM Tris-HCl pH 8.5 containing 1% (w/v) BSA (0.25 g) and a 1: 5 000 dilution of extravidin peroxidase (4 µl). Plates were incubated at 37°C for 45 minutes and then washed as previously described but replacing the final wash with 100mM sodium acetate pH 6.0. The plates were developed with 200.0 µl per well of developing buffer (150 µl of 10mg ml^{-1} TMB and 3 µl of 30% (v/v) hydrogen peroxide were added to 20.0 ml of 100 mM sodium acetate pH 6.0). The colour development depends on the amount of transglutaminase in each well and the development time. The colour was developed for 15 minutes. Colour development was terminated by the addition of 50.0µl per well of 5.0M sulphuric acid (H_2SO_4) at exactly 15 minutes. The absorbance was read at 450nm using a Titertek Multiscan ELISA Spectrophotometer. One unit of transglutaminase activity can be defined as a change in absorbance at 450nm of 1.0 per hour.
After the preparation of 10% SDS- PAGE gels; 2µl of standard protein, 4µl of standard TG-ase with 10.0µg each of crude, dialysed and purified protein sample were pipetted into the gels. The gel cassette container is then filled with running buffer pH 8.3 (10x stock) [6.0g Tris, 28.4g glycine dissolved in 1.0 litre with 0.1% (w/v) SDS]. The gels were run at 80ma and the voltage was adjusted such that it does not exceed 200volts. The experiment was stopped immediately the standard protein reaches the 0.5cm from the end of the gel. The gels were removed and one of it was stained with blue stain (instant blue, a coomassie based staining solution for protein gel) for 2 hours on shaking. The gel was washed with distilled water for several times in order to achieve a clear background. Photograph of it was taken with Genesnap.
The proteins were separated by gel electrophoresis and a western blot procedure (Towbin *et al*., 1978) was conducted. The membrane was incubated with appropriate dilution of primary antibody (2:1000) – 70.0 µl of anti-TGase antibody (ID10) in 35.0 ml PBS-Tween 80 for 2 hours at room temperature on shaking. The unbound primary antibody was removed by washing the probed blot in PBS/Tween 6 times, 10 minutes each making a total washing time of 1 hour. The blot was then incubated with goat serum for another 2 hours on shaking at room temperature so that the secondary antibody can react with the plant protein; this was also followed by 6 times washing 10 minutes each with PBS/Tween 80. The probed nitrocellulose membrane was later incubated with secondary antibody (Anti- mouse IgG (ɣ-chain specific) peroxidase, developed in Goat (2:5000) - 16µl in 40.0ml PBS/Tween for 2 hours at room temperature on shaking. It was washed as described earlier to remove unbound secondary antibody. The membrane was developed to reveal antibody-antigen binding using the following substrate mixture; 33.0 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 50 mg/ml in dimethyl formamide [DMF]), 44 µl Nitroblue tetrazolium (NBT; 75 mg/ml in 70% v/v DMF) in 20.0 ml substrate buffer. Colour development took up to 30 minutes, the developed blot was rinsed in distilled water and blot dry on filter paper. The photograph was taken with Genesnap.
Alternatively, antibody reactivity was detected using enhanced chemiluminescence (ECL). In this case, the washed and probed blot was placed on a clean glass plate and excess buffer removed with filter paper. A 1:1 mixture of Amersham ECL reagents A+B (approximately 2 ml total volume) was spread evenly on the surface of the blot and left for 1 minute at room temperature. The blot was then exposed for 30 seconds onto hyper film ECL and the reactivity was revealed.

Results

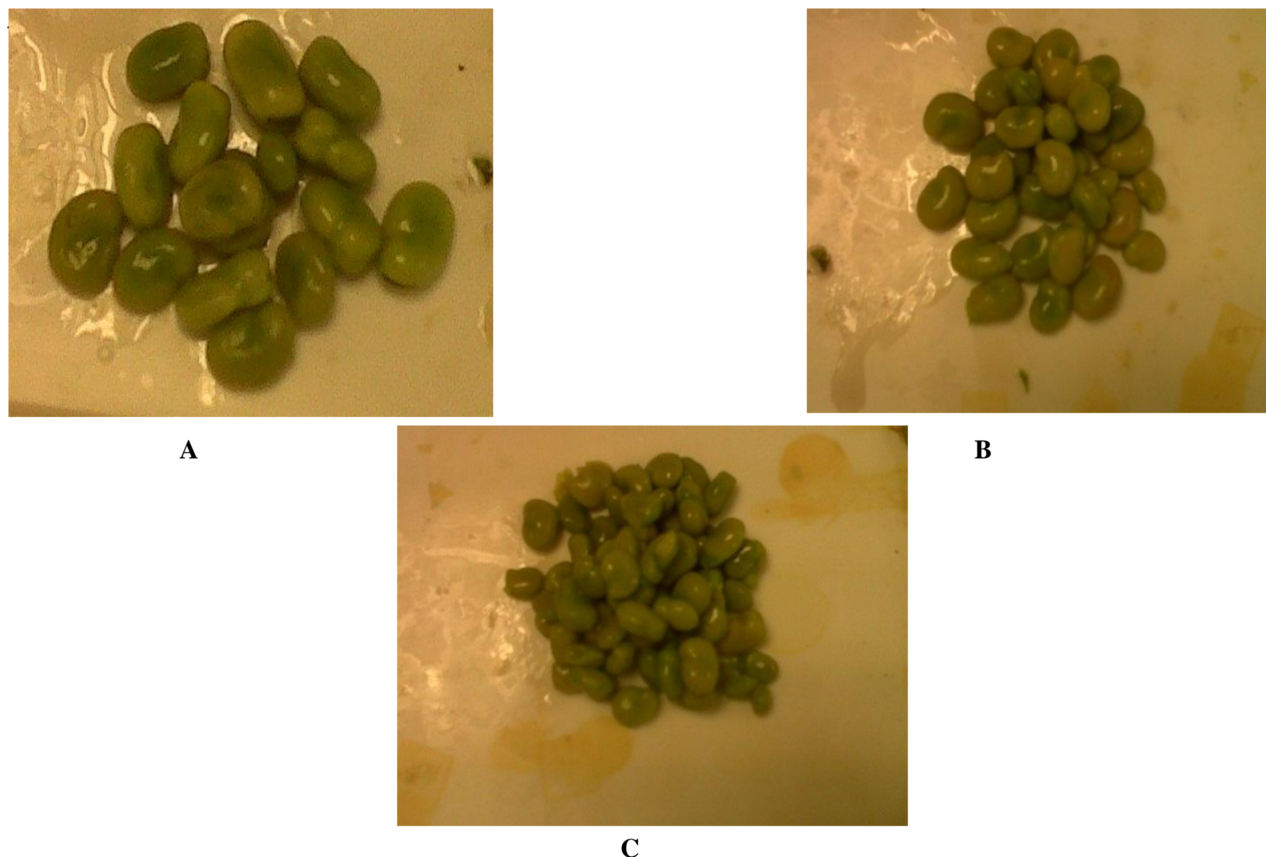


Figure 1: Seeds of broad bean was harvested from the pods and were divided into three different group based on the sizes. A. Large seeds with the average dimension of 2.0 cm long, 1.5cm broad and 1.0 cm thick. B. Medium seeds with the average dimension of 1.3 cm long, 1.0 cm broad and 0.7 cm thick. C. Small seeds with the average dimension of 1.0 cm long, 0.7 cm broad and 0.4 cm thick.

Table 1: Detection of transglutaminase in crude and dialysed fractions of extract from mature *Vicia faba* cotyledon

Samples	Total Protein mg	Specific activity Units mg-^{1}	Total activity Units
Crude (1:10 dil.)	9.6	0.329 ± 0.009	3.158 ± 0.09
Crude (1:50 dil.)	10.46	0.224 ± 0.010	2.343 ± 0.10
Crude (1:250 dil.)	15.15	0.189 ± 0.012	2.863 ± 0.18
Dialysed (1:10 dil.)	6.3	0.269 ± 0.015	1.695 ± 0.09
Dialysed (1:50 dil.)	7.0	0.178 ± 0.004	1.246 ± 0.03
Dialysed (1:250 dil.)	17.25	0.172 ± 0.012	2.967 ± 0.207

Table 1 Transglutaminase activity was measured in the 1:10, 1:50 and 1:250 dilutions of crude and dialysed cotyledon extracts of *Vicia faba* using biotin cadaverine incorporation assay, 6.67mM $CaCl_2$ was replaced with 1.33mM EDTA as a control. Data points represent the mean ± SEM of 4 replicates.

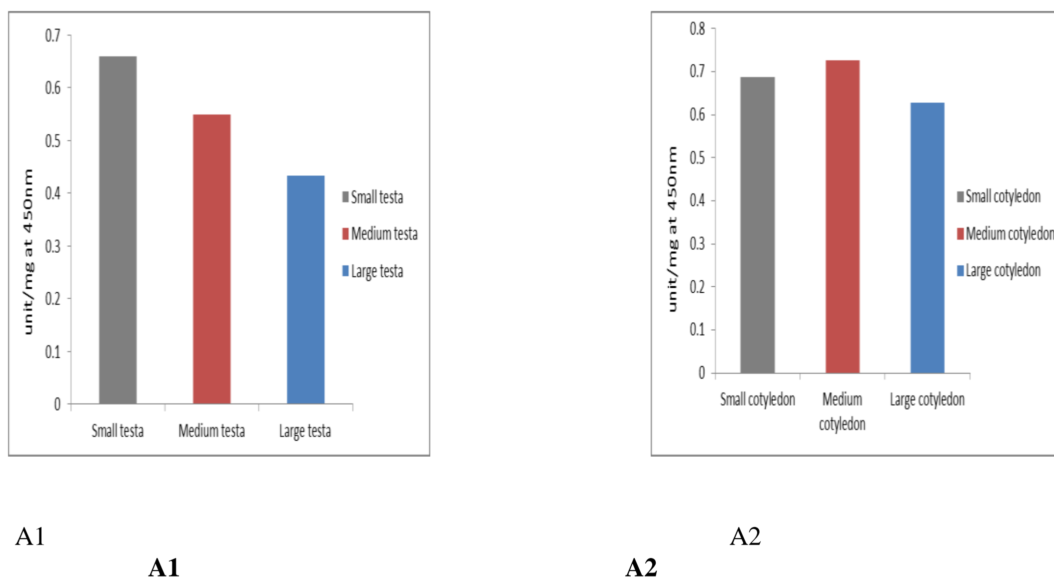
Table 2: Detection of transglutaminase in the extract from the testa and cotyledon of *Vicia faba* seeds at different stages after development.

Samples	Total Protein mg	Specific activity Units mg^{-1}	Total activity Units
Small testa	30.87	0.660 ± 0.039	20.37 ± 1.203
Medium testa	30.73	0.548 ± 0.037	16.84 ± 1.137
Large testa	20.28	0.433 ± 0.009	8.78 ± 0.183*
Small cotyledon	13.45	0.687 ± 0.040	9.24 ± 0.538
Medium cotyledon	13.11	0.727 ± 0.176	9.53 ± 2.307
Large cotyledon	11.52	0.628 ± 0.097	7.23 ± 1.117

*means are significantly different at p≤0.05

Table 2 Transglutaminase activity of testa and cotyledon was measured in the small, medium and large size seeds of *Vicia faba* using biotin cadaverine incorporation assay (section 2.4.2). 6.67mM $CaCl_2$ was replaced with 1.33mM EDTA as a control. Data points represent the mean ± SEM of 3 replicates.

Figure 2: Extracts from either testa or cotyledon of broad bean seeds at different stages after development was assayed for transglutaminase activity. Data points represent the mean ± SEM of 3 replicates (value plotted is that obtained in the presence of calcium, minus the value obtained from the EDTA-containing parallel control well).

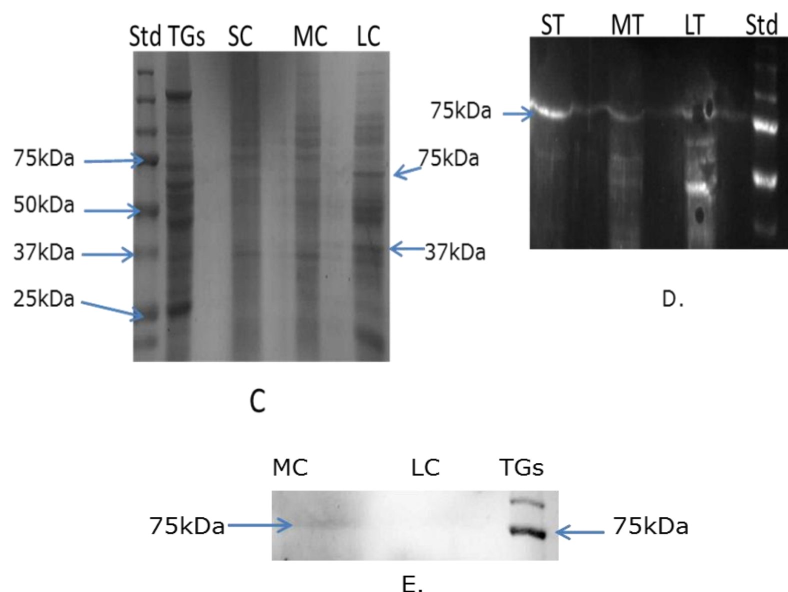


A1 A2

A1 **A2**

DescriptionAboutfigure 2: A2 shows that extract from the cotyledon of medium size seeds of *Vicia faba* gave the highest transglutaminase specific activity of 0.727 units/mg followed by the extract from the small size seeds giving a specific activity of 0.687units/mg and extract from the cotyledon of large size seeds shows the least value of transglutaminase specific activity of 0.628 units/mg. A1 shows that extract from testa of small size broad bean gave the highest value of specific activity of 0.660 units/mg, followed by medium size with a value of 0.548 units/mg and extract from the testa of large size seeds of *Vicia faba* gave the least value of 0.433 units/mg.

Figures 2C, 2D and 2E: **SDS-PAGE of the extract from the cotyledon of *Vicia faba* at different stages after development, and the western blot of the extract from the testa and cotyledon of the seeds of *Vicia faba*.**



DescriptionAboutFigures 2C, 2D and 2E: for figure 2C; electrophoresis of cotyledon extract from *Vicia faba* seeds at different stages after development (large, medium and small stages for both testa and cotyledon) were performed. While in figure 2D and 2E; western blot protocol was conducted for the extract from testa and cotyledon of the seeds of *Vicia faba* at different stages after development.In 2C, the extract from the cotyledon shows the 37 kDa protein band. Figure 2D and 2E show that the extract from the cotyledon of medium size seeds and the testa (small, medium and large seed testa) has the 75 kDa protein band which is also present in tissue transglutaminase from guinea pig liver.

* Std= Molecular mass standard, TGs= standard transglutaminase from guinea pig liver, SC= small cotyledon, MC= medium cotyledon, LC= large cotyledon, ST= small testa, MT= medium testa and LT= large testa. C, coomassie staining of testa and cotyledon (10µg protein). D and E, primary antibodies dilution 2:1000; goat serum 2:1000 and secondary dilution 2:5000.

Table 3: Evidence of crosslink in various samples
Standard Tgase area= 12205421

Samples	Crosslink area	nmol crosslink
Control	ND	ND
Small testa	ND	ND
Medium testa	392480	32.2
Large testa	952158	78.01
Small cotyledon	972373	79.66
Medium cotyledon	943562	77.30
Large cotyledon	ND	ND
Mature cotyledon	-----	-----

*ND=Not detected, the X link (cross link) of each sample was derived by dividing the area of crosslink of the sample by the area of crosslink of standard Ɛ-(ɣ-glutamyl) lysine.

i.e. μmol X link = $\frac{\text{Area of crosslink for sample}}{\text{Area of crosslink for standard Ɛ-(ɣ-glutamyl) lysine}}$

Table 3: The values of crosslink of the samples based on 100.0 μmol total amino acid were computed by dividing the area of crosslink of the standard transglutaminase by each crosslink area of samples.

DISCUSSION AND CONCLUSION

The results presented above provides some evidences that transglutaminase2 activity is present in the cotyledon of mature broad beans and as well present in both the testa and the cotyledon of the other three different stages after broad bean seed development. The results also show the presence of Ɛ-(ɣ-glutamyl) lysine isodipeptide bonds in plant tissues connoting the actual evidence of transglutaminase activity.

Transglutaminase activity

The strength with which plant extracts use to incorporate N'N'-dimethylcasein into protein has been observed over the years (Serafini-Fracassini *et al*., 1988, Aribaud *et al*., 1995 referenced in Lilley *et al*., 1998). Table 1 shows transglutaminase activity in crude and dialysed extract from the cotyledon of mature broad bean (*Vicia faba*). The table revealed that the activity was higher in the crude fraction than the dialysed fraction. Also since transglutaminase activity was detected using the biotin-cadaverine incorporation assay of Slaughter *et al*., (1992), the activity in the cotyledon of mature broad bean is not affected by the assay. However, this suggests that the use of biotin cadaverine assay is perfect for the study of transglutaminase from crude sample of plant cell extract (Lilley *et al*., 1998). The highest value for specific activity from the mature cotyledon was from the crude fraction and it gave 0.329 Units mg^{-1}.

Furthermore, table 2 shows the transglutaminase activity in the testa and the cotyledon of *Vicia faba* at different stages (sizes) after development. The highest value of specific activity in the testa was found in the extract from the testa of small size seeds fraction giving a value of 0.660 Units mg^{-1}, followed by the testa of the medium size seeds giving 0.548 Units mg^{-1} and decreased to 0.433 Units mg^{-1} in the testa of the large size seeds fraction. In addition to this, the extract from the cotyledon of small size seeds gave a specific activity of 0.687 Units mg^{-1}, which later increased in the cotyledon of medium size seeds giving a value of 0.727 Units mg^{-1} and the specific activity eventually decreased as the seeds get bigger giving a value of 0.628 Units mg^{-1}. Comparing the highest value in the mature cotyledon (0.329 Units mg^{-1}) from (table 1) with those of table 2; transglutaminase activity in the different stages of seeds of broad bean after development), it appears that all the values for the specific activity of the cotyledon at different stages after development are higher than those of the mature cotyledon (table 1). This might be as a result of the alleged involvement of transglutaminase in cell growth, cell differentiation and cell development (Kaczkowski, 2005). As the seeds get mature, the transglutaminase content keeps decreasing. However, the specific transglutaminase activity present in the cotyledon is higher than that of the testa (table 2). This might also be as a result of its assumed involvement in the strengthening of cell wall concurring with the findings of Del Duca *et al*., (2007) which states that the cell wall transglutaminase have a role in strengthening the corolla cell wall of *Nicotiana tabacum* by structurally modifying the cell wall through protein cross-linking.

Transglutaminase location:

The 75 kDa band is the most abundant band, being detected in the testa of small size seed, medium size seeds, large size seeds (figure 2 D) and also came out faint in the cotyledon of medium size seeds fraction (figure 2E). The 50 kDa band which is expressed only in the testa of large size seeds fraction might have being derived from the 75 kDa enzyme by post-translational modification taking place in the cell wall (Del Duca *et al*., 2007).

However, goat serum was used after the first probe with primary antibody (anti-TGase antibody [ID10]), this was done in order to increase the chances of the antigen-antibody reactivity of the secondary antibody (ɣ-anti-mouse IgG [-chain specific] peroxidase, developed in goat) with the plant protein.

Crosslink

The detection of ε-(γ-glutamyl) lysine isodipeptide bonds which is the authentic proof that transglutaminase2 is active in tissues was carried out in the testa and cotyledon compartments of the different stages of *Vicia faba* seeds after development using an amino acid analyzer (System Biochrom 30 Flu). The crosslink for the standard ε-(γ-glutamyl) lysine isodipeptide was used as the bases for the detection of the crosslink of the samples (i.e. extracts from the testa and cotyledon of small, medium and large size seeds of broad bean after development respectively). Crosslink was found present in the protein digestion from the testa of medium and large size seeds; cotyledon of small and large size seeds, and was not detected in testa of small size seed and in the cotyledon of large seeds of *V. faba* (Table 3).

Plant transglutaminase portray a range of similar characteristics with those of animal Transglutaminases, despite a different configuration in their amino acid sequence except for the Cys-His-Asp conformation known as the catalytic triad. Though transglutaminase 2 is present in very low amount in the broad bean seeds, but its presence in the testa and cotyledon of the seeds shows that it is responsible for lot of functions and it is very essential since it can be related with different processes taking place within or outside of cell. However, more work needs to be done on this particular species of plant, in order to confirm the specific functions of transglutaminase 2 (TG2) in the testa and the cotyledon. The total amino acid from various compartments hydrolysed with 6N HCl will be compared with the crosslink content of the standard ε-(γ-glutamyl) lysine in future work for the confirmation of the presence of glutamate and lysine in the tissue. Also, the work will be extended to *Pisum sativum* (Pea) which is in the same family with broad bean (Fabaceae) in the plant kingdom. The presence of transglutaminase 2 in another family member of fabaceae such as *P. sativum* can then drive to the general conclusion that transglutaminase 2 is present and active in all members of the family of fabaceae. Also attempt of investigating the specific function of this enzyme "Transglutaminase 2" in some other monocotyledonous and dicotyledonous plants will be done.

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