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BRIEF COMMUNICATION

Concurrent occurrence of α-amylase inhibitor and stimulator in red kidney bean seed: physiological implications

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Abstract

It is hypothesized that since protein α-amylase inhibitor (α-AI) and stimulator might be present together in red kidney bean (*Phaseolus vulgari*s L.) seeds, their *in vitro* interactions might influence their detection and quantification. Assay of α-AI using extracts from the embryonic axes revealed an unexpected finding in that the extracts stimulated rather than inhibited α-amylase activity. The cotyledon extracts exhibited inhibitory or enhancement effect on α-amylase activity depending on whether prior to the α-amylase assay they had been boiled for 10 min or not. Phytohemagglutinin (PHA-L in particular) is implicated in the present study as a stimulator of α-amylase activity co-extracted with α-AI from red kidney bean cotyledons. The importance of these findings is discussed in relation to the possible widespread occurrence of protein α-amylase stimulator in seeds and other plant parts.

Additional key words: lectins, *Phaseolus vulgaris*, phytohemagglutinins.

Regulation of α-amylases in seeds has been the focus of a lot of research (Ashraf *et al*. 2002; Sodkiewicz and Sodkiewicz 2003, Kato-Noguchi and Macias 2008). Interestingly, dry mature red kidney bean seeds (*Phaseolus vulgaris*) contain biologically active proteins including α-amylase inhibitors (α-AI) against insect and mammalian α-amylases but not plant α-amylases (Santimone *et al*. 2004). Porcine pancreatic α-amylase (PPA), a commercially available mammalian α-amylase, is widely used to facilitate determination and purification of α-amylase inhibitors in extracts of seeds (Payan 2004, Santimone *et al.* 2004). Another group of abundant biologically active seed proteins in *P. vulgaris* consists of lectins, particularly phytohemagglutinin (PHA). The five tetrameric isoforms of PHA are mainly comprised of combinations of two types of subunits, the PHA-E and PHA-L (Morai *et al.* 2008).

The α-AI and PHA from *P. vulgaris* have been shown to be anti-insect proteins (Kluh *et al.* 2005) and mitogens of lymphocytes (Shi *et al.* 2007), respectively. However, their *in vivo* functions apart from being a part of the nitrogen store for seed germination remain unclear (Morari *et. al.* 2008). A previous report showed that PHA-E and PHA-P purified from *P. vulgaris* were able to enhance α-amylase activity *in vitro* (You and Chang 1992). However, the implications for α-AI and PHA-L (or other isoforms of PHA) present together in the same seed extracts and their possible *in vitro* interactions on the detection and quantification of α-AI have not been the specific focus of any previous studies.

Here, we report that PHA-L is able to enhance α-amylase activity *in vitro*. Moreover, when PHA was present together with α-AI in the same bean seed extract, detection and interpretation of assay results on inhibitor (α-AI) and stimulator (PHA) of α-amylase activity was complex, a situation likely unrealized in previous work. The occurrence of protein α-amylase stimulator in seeds and possibly other plant parts as well could be widespread. Establishing this is an important prerequisite for designing future investigations to gain new insights into the little known physiological significance of protein α-amylase stimulator at least in red kidney bean seeds.

Seeds (7 g) of red kidney bean (*Phaseolus vulgaris* L.) purchased from a local health food store were soaked in distilled water (20 cm^3) in a beaker for 15 h at room temperature. Then cotyledons and embryonic axes were isolated from the seeds and extracts were prepared following the method of Le Berre-Anton *et al*. (1997).

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Abbreviation: α-AI - α-amylase inhibitor; PHA - phytohemagglutinin.
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Aliquots of the extracts were used for detection of α-AI activity based on the method of Moreno *et al*. (1994). Additionally, in one experiment, a purified leuco-agglutinin (PHA-L) of *P. vulgaris* (*Sigma*, St. Louis, USA) was used instead of bean protein extracts in the α-AI assay using PPA. The cotyledon extracts prepared were also purified using a porcine thyro-globulin-agarose (*Sigma*) column (1 × 12 cm) following the method of Ren *et al.* (2008a). Protein content in bean seed extracts were quantified using a method based on quantitative binding of proteins with the Coomassie brilliant blue dye (Bradford 1976). Bovine serum albumin was used as a standard.

The extracts obtained from red kidney bean cotyledons were fractionated with ammonium sulfate and the fraction obtained between 0 - 85 % saturation was applied to an anionic exchange DEAE cellulose column equilibrated with 20 mM phosphate buffer (pH 6.8) and subsequently the fraction with unbound proteins from this column was applied to an affinity column (*Affi-Gel Blue* gel, *Bio-Rad*, Hercules, USA) equilibrated with 20 mM Tris buffer (pH 7.3). A bound protein of apparent M_r of 31 - 33 kDa in a fraction from the *Affi-Gel* column was sent to the Centre for Protein Research, Department of Biochemistry, Otago University (Dunedin, New Zealand) for MALDI mass spectrometric analysis.

All experiments were repeated at least three times and there were three replications for each reaction mixture. Data were subjected to one-way analysis of variance (*ANOVA*, $P \leq 0.05$) followed by comparison of means using Duncan's Multiple Range test at 5 % level of significance (Clewer and Scarisbrick 2001).

Only cotyledon or whole seed extracts have been commonly used for studies of α-AI. There is a paucity of information about the effect of extracts from the embryonic axis on α-amylases. An unexpected, new result was obtained when extracts from red kidney bean embryonic axes were used in the α-AI assay. α-Amylase activity in the presence of these extracts was higher than in their absence even though they did not contain any detectable starch hydrolyzing activity (Table 1). In contrast, after electrophoresis of the embryonic axis extracts through a non-denaturing polyacrylamide gel several isoinhibitors of α-amylases were clearly present (Alizadeth, Leung and Cole, unpublished results).

When the cotyledon extracts were used in the α-AI assay, the presence of α-AI was clearly evident (Table 2). However, after the cotyledon extracts had been placed in a boiling water bath (100 ± 2 °C) for 10 min, a treatment found to be effective in the loss of isoinhibitor bands of α-amylase (Alizadeth, Leung and Cole, unpublished), the enhancing effect of the extracts on α-amylase was revealed (Table 2). This finding is consistent with that of You and Chang (1992) when whole red kidney bean seeds were homogenized to prepare the extracts.

From the results it would appear there may be a factor present in red kidney bean seeds that is capable of enhancing α-amylase. Detection of the factor depends on its activity relative to that of the α-AI (masking α-AI in the embryonic axis extracts) or its separation from the α-AI (after boiling or further purification of the cotyledon extracts).

Table 1. Enhancement of α-amylase activity by embryonic axis extract (0.1 μg of protein). Control reaction was without α-amylase and embryonic axis extract and therefore a high absorbance reading (A_{620}) of around 1.68 was obtained. Each assay was carried out in triplicate. Mean absorbance values ± SE assigned with the same letter do not differ significantly according to DMRT ($P < 0.05$).

Treatments	Amylase activity
Amylase (PPA)	0.876 ± 0.016b
Amylase + embryonic axis crude extract	0.247 ± 0.013a
Embryonic axis crude extract	1.696 ± 0.170c
Control	1.682 ± 0.023c

Table 2. Effect of red kidney bean cotyledon extract (1 μg of protein) on α-amylase activity before and after boiling, and in a separate experiment studying effect of PHA (7 μg) purified from red kidney bean cotyledons on α-amylase activity. Control reaction was without α-amylase in the first experiment or PHA in the second experiment. The control reactions resulted in the high absorbance readings (A_{620}) of 1.162 and 1.316 in the respective experiments. Each assay was carried out in triplicate. Mean absorbance values ± SE assigned with the same letter do not differ significantly according to DMRT ($P < 0.05$).

Treatments		Amylase activity
Cotyledon extract	amylase (PPA)	0.650 ± 0.015b
	amylase + boiled extract	0.240 ± 0.005a
	amylase + extract	1.022 ± 0.028c
	boiled extract (no PPA)	1.165 ± 0.020d
	extract (no PPA)	1.160 ± 0.015d
	control	1.162 ± 0.021d
PHA	amylase (PPA)	0.662 ± 0.031b
	amylase + PHA	0.214 ± 0.015a
	PHA	1.296 ± 0.007c
	control	1.316 ± 0.015c

It has been reported that purified PHA from *P. vulgaris* seeds can promote α-amylase *in vitro* (You and Chang 1992). The stimulator revealed in the present study could also be a PHA. Purification of the cotyledon extracts using an affinity column yields a single band of apparent M_r of approximately 32 kDa on SDS-PAGE (data not shown). The protein (7 μg) in the fraction collected from the affinity column is devoid of amylase or α-AI but instead it enhanced the α-amylase activity by close to 70 % (Table 2). Since in a previous study by You and Chang (1992), purified PHA-L was not used to test for enhancement of α-amylase activity, a commercial PHA-L (*Sigma*) was used in an experiment similar to that described in Table 2. The commercial PHA-L had no detectable amylase activity but was also found to be capable of promoting α-amylase activity (by about 80 %

brought about by addition of 7 μg of purified PHA-L, data not shown). Using 300 μg of other subunits of PHA including PHA-P, less than 50 % increase in α-amylase activity was observed (You and Chang 1992).

To verify that PHA-L was indeed present in the low-pH cotyledon extracts that also contain α-AI, another protein purification experiment independent of thyroglobulin-agarose column chromatography was carried out. Bean cotyledon extracts containing α-AI activity (prepared in the same way as those used in the experiment as described in Table 2A) were partially purified using DEAE-cellulose followed by *Affi-Gel Blue* column chromatography. Then a fraction containing a protein of apparent M_r of approximately 31 - 33 kDa had been identified to be PHA-L after MALDI mass spectrometry (data not shown).

Similar to the enhancing effect of PHA on α-amylase activity, a leaf lectin when added to insect gut homogenates was shown to enhance the insect gut α-amylase activity by Macedo *et al* (2007). Therefore, it would seem possible that the occurrence of proteins in plants that can enhance animal α-amylase activities might be widespread and is worthy of further study. It would be also of interest to determine if plant proteins other than lectins posses an enhancing effect on α-amylase activity.

In conclusion, the co-presence of α-amylase inhibitor and stimulator in plant extracts might be widespread. Their detection and quantification could be complex but this has likely been overlooked in the previous studies on α-amylase inhibitor at least from red kidney bean seeds. This complexity might also have contributed to the paucity of knowledge about the physiological significance, if any, of protein α-amylase stimulator in seeds. Further studies are now underway along this new line of investigations.

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