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Starch Blocking Stability of the *Phaseolus vulgaris* Alpha-Amylase Inhibitor (α-AI1)

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Abstract: The starch blocking stability of speckled kidney beans (*Phaseolus vulgaris*) alpha-amylase inhibitor (α-AI1) for application as a nutraceutical additive against diabetes and obesity was assessed. The inhibitor was purified to 0.09% w/w of seed flour using pH fractionation, alcohol precipitation (75%), DEAE-Sepharose CL-6B and Sephacryl S-200 chromatography. The interactive effect of pH (A), temperature (B) and time (C) on residual inhibitory activity was modeled using Response surface methodology with the Box-Behnken design. Intrinsic fluorescence and ANS-assisted surface hydrophobicity indicated activity loss is accompanied with tertiary structural unfolding. Chaotrophic salts at high (1.0 M) and kosmotrophic salts at low (0.1-0.01 M) concentration stabilized the inhibitor in the order $CH_3COO^- > Cl^- > Br^- > I^- > SCN^-$ and vice-versa, respectively.

Key words: *Phaseolus vulgaris* alpha-amylase inhibitor (α-AI1), heat stability, diabetes, obesity, hofmeister series salts, surface hydrophobicity

INTRODUCTION

Phaseolus vulgaris α-amylase inhibitor (α-AI1) extracts, known as starch blockers recently have been developed into more effective control agents for obesity and diabetes (Meiss *et al*., 2004). A starch blocker is a substance that interferes with the breakdown of starch leading to reduced digestibility or prolonged digestion such that energy derived from the starch is reduced or the rate of body absorption of the energy in form of glucose is reduced (Celleno *et al*., 2007). Some *Phaseolus vulgaris* alpha-amylase inhibitor (α-AI1) starch blocker products have been considered and approved for GRAS status by the USDA (Chokshi, 2006). The *Phaseolus vulgaris* α-amylase inhibitor (α-AI1) starch blockers have been shown through several animal and human studies to significantly reduce postprandial hyperglycemia and to at least cause subtle weight loss without any observable adverse effects on subjects (Celleno *et al*., 2007; Tormo *et al*., 2004; Udani *et al*., 2004). Due to their protenaceous nature and activity profile, for effective *in vivo* anti-amylase activity, the *Phaseolus vulgaris* alpha-amylase inhibitor (α-AI1) starch blockers should be taken before or with starch containing meals (Le Berre-Anton *et al*., 1998; Santimone *et al*., 2004; Obiro *et al*., 2008). Incorporation of the inhibitor as an ingredient in food products would therefore ensure appropriate and effective utilization.

Though the *Phaseolus vulgaris* α-amylase inhibitor (α-AI1) has potential as a nutraceutical additive, relevant information on its functionality that is relevant application is limited (Obiro *et al*., 2008). Its application in foods as an ingredient before heat processing, though convenient, could expose it to potential loss of activity through denaturation. On the other hand, the inhibitor has been shown to be variably stable between 40-90°C (Collins *et al*., 2006; Sawada *et al*., 2001; Yoshikawa *et al*., 2000).

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The variation of the inhibitory activity as affected by pH, temperature and heating time as would occur in different food systems however has, to the best of our knowledge, never been assessed. In addition no attempt has been made to investigate heat treatment related aspects such as effect of salts and polyols on its stability and the molecular conformational changes related to its inactivation. In this study therefore, the starch blocking stability of a purified α-amylase inhibitor (α-AI1) from red speckled kidney beans, was characterized using response surface methodology with respect to temperature, pH and heating time. The underlying tertiary conformational changes that accompany loss of inhibitory activity were examined using intrinsic fluorescence, UV absorbance and anilino-1-naphthalenesulfonic acid (ANS)-assisted surface hydrophobicity (Ho). The residual inhibitory activity, as affected by selected Hofmeister series salts (CH_3COO^-, Cl^-, B^-, I^-, SCN^-) and polyols (ethylene glycol, glycerol, sorbitol and polyethylene glycol-2000) was investigated too. The results obtained here will lay a foundation for the pre-heat treatment application of the *Phaseolus vulgaris* alpha-amylase (α-AI1) starch blockers as nutraceutical additives in different food systems, thereby providing a convenient aid against obesity, diabetes and related conditions.

MATERIALS AND METHODS

Materials

The speckled kidney bean variety Huayundou, which is widely consumed in China, was used. Dried beans were obtained from the local market in Wuxi, People's Republic China. Porcine Pancreatic Amylase (PPA) type B IV and 8-Anilino-1-naphthalenesulfonic Acid Ammonium Salt (ANS) were obtained from Sigma-Aldrich (St Louis, USA). Chromatography gels were obtained form GE Healthcare Life Science (Piscataway-NJ, USA). All other reagents were of analytical grade and were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Phaseolus vulgaris Alpha-Amylase Inhibitor (α-AI1) Purification

Crude extraction of the inhibitor was done according to Pusztai *et al*. (1995), while chromatographic purification was done according to Sawada *et al*. (2001) with modifications. Ion exchange chromatography was done using DEAE-Sepharose CL-6B equilibrated with 20 mM Tris-HCl buffer at pH 7.1. An appropriate amount of crude extract (approximately 0.3 g) was dissolved in 50 mL of buffer then stirred overnight at room temperature. The solution was then centrifuged at 8000 x g, for 3 min at 4°C before loading on to the column. The column was washed with excess of loading buffer, until the UV-detector reading at 280 nm was below 0.005. The elution was done using 0-0.5 M NaCl gradient in the Tris-HCl buffer over 1000 mL at a flow rate of 2 mL min^{-1}. Fractions (10 mL) were collected and assayed for inhibitory activity. The fractions with the highest alpha-amylase inhibitory activity were combined. Gel filtration was done using a Sephacyl S-200 column equilibrated with the 20 mM, pH 7.1 Tris-HCl buffer and the protein was eluted with the same buffer. The first pronounced peak was collected. Ultrafiltration was then done using a 10,000 molecular weight cut-off membrane bench-top stirred cell system (Millipore Corp., Bedford, MA). The retentate was collected as the purified inhibitor and stored at -20°C until further use. The protein content of the purified α-amylase inhibitor solution was determined according to the Lowry *et al*. (1951) method with BSA as the standard. SDS-PAGE was done on 12% separating and 4% stacking gels according to Laemmili (1970) using low molecular weight (14400-974000) markers obtained from Sigma-Aldrich (St. Louis, MO). Hemagglutinating activity was measured according to Vasconcelos *et al*. (1991) using human blood (group O, Rhesus +ve). Specific hemagglutinating activity was defined as hemagglutinating units per milligram of total analytical sample protein.

Porcine Pancreatic Alpha-Amylase and Inhibitor Activity

Alpha amylase activity and inhibitory activity were determined according to Le Berre-Anton *et al*. (1997) with some modifications. To a soluble starch solution (0.4 mL, 1% w/v) in 80 mM

phosphate buffer (pH 6.9), PPA (0.2 mL, 0.001% w/v) in 20 mM acetate buffer (pH 4.5 containing 20 mM $CaCl_2$ and 10 mM NaCl) was added and then incubated for 15 min at 37°C. The final pH of the total reactants was 5.5 after initiation of the reaction. The reaction was stopped by addition of 0.8 mL of dinitrosalicylic acid reagent (6 g L^{-1}). This solution (1 mL) was boiled for 5 min, then cooled immediately on ice and then diluted with 4 mL of water. Absorbance was read at 540 nm. Appropriate blanks were prepared without PPA. A maltose standard curve was drawn to enable determination of the amount of maltose produced. Assays for the α-amylase inhibitor activity were performed as described above but the PPA solution and purified inhibitor solutions (0.2 mL) were pre-incubated for 15 min before addition of the soluble starch solution. Alpha-amylase inhibitory activity was determined according to Eq. 1 shown below:

$$\text{Inhibitory activity (\%)} = \left(\frac{\text{Mo} - \text{Mi}}{\text{Mo}}\right) \times 100 \quad (1)$$

where, Mo and Mi are the amount of maltose (mg mL^{-1}) produced in absence and presence of the inhibitor, respectively, under the same conditions.

Heat Stability Treatments

Heat treatments were done following the method by Makki and Durance (1996) with some modifications. The buffers used for pH adjustment were; citrate (pH 3.5), citrate-phosphate (pH 5.5) and, phosphate (pH 7.5) and were of the same concentration (10 mM). Stock solutions of the inhibitor were obtained by dissolving 1 mL of the purified alpha-amylase inhibitor in 9 mL of appropriate buffer at each pH. For each heat treatment, 9 mL of the appropriate buffer was sealed in a capped 10 mL capacity glass vial and incubated in an agitated water bath set at the test temperature for 20 min. The inhibitor stock solution (1 mL) was then injected into the vial giving a final concentration of 0.015 mg mL^{-1} and then recapped. For each time/temperature combination, the sample (0.2 mL) was taken from the vial and transferred immediately into a microfuge tube in a water/ice bath to cool. Samples were assayed for residual inhibitory activity either the same day, or stored at 4°C until assay time.

For effects of selected Hofmeister series salts and polyols, sodium of CH_3COO^-, Cl^-, Br^-, SCN^- and I^- were used while the polyols used included glycerol, ethylene glycol, polyethylene glycol and sorbitol at concentrations of 10.0, 1.0 and 0.1% (w/v) each. The salts were dissolved in phosphate buffer (0.1 M, pH 7.5) at concentrations of 0.001, 0.10, 1.0 M and the control had no salts added. The inhibitor solution (0.1 mL, 1.5 mg mL^{-1}) was dissolved in the buffer (0.9 mL) to obtain the analytical samples. These samples were incubated at 75°C for 25 min and then cooled immediately in an ice/water bath. Tests for residual inhibitory activity were performed in triplicate.

Intrinsic Fluorescence and Ultraviolet Absorption Spectra

The samples were heat treated as described above at 75°C, in 10 mM phosphate buffer (pH 7.5) for 0, 5, 15, 25 and 45 min. The fluorescence spectra were determined with a Hitachi 650-60 spectrofluorometer (Tokyo, Japan). A slit diameter value of 5 nm was used for both the excitation and emission slits. The excitation wavelength was 280 nm and the scan ranged between 300 and 360 nm. UV-spectra scans were recorded from 250-300 nm with a Shimadzu UV2450 spectrophotometer (Tokyo, Japan).

Surface Hydrophobicity Measurements

Surface hydrophobicity (Ho), was determined using the 8-Anilino-1-naphthalenesulfonic acid (ANS) probe on the Hitachi 650-60 spectrofluorometer (Tokyo, Japan). Stock solutions of the protein

Table 1: Selected factor levels

			Levels		
Factors	Symbol	Units	High (1)	Center (0)	Low (-1)
pH	A		7.5	5.5	3.5
Temperature	B	°C	60.0	75.0	90.0
Time	C	min	5.0	15.0	25.0

Table 2: Statistical design (Box-Behnken) with resultant responses

Runs		Factors (coded)			Response	
Standard order	Randomized order	A: pH	B: Temp (^{0}C)	C: Time (min)	Residual activity (% inhibition)[a]	
17	1	0	0	0	96.8	93.2
15	2	0	0	0	95.8	92.2
11	3	0	-1	1	95.2	96.5
1	4	-1	-1	0	90.4	89.3
2	5	1	-1	0	97.1	95.5
16	6	0	0	0	95.9	93.2
12	7	0	1	1	4.0	4.8
8	8	1	0	1	17.8	16.8
5	9	-1	0	-1	47.1	45.1
13	10	0	0	0	97.8	91.2
4	11	1	1	0	7.6	4.6
9	12	0	-1	-1	94.4	95.7
6	13	1	0	-1	63.8	65.9
10	14	0	1	-1	9.2	8.7
3	15	-1	1	0	8.1	6.8
7	16	-1	0	1	28.0	26.2
14	17	0	0	0	94.8	90.2

[a]Each run value is an average of triplicates

and ANS were prepared in 10 mM phosphate buffer (pH 7.5) at concentrations of 0.015% (w/v) and 8×10^{-3} M, respectively. Analytical protein solutions were then obtained by addition of 30 µL of the ANS stock to 2 mL of protein solutions at 0.001-1.5% w/v. ANS was not added to the control solutions. Fluorescence intensity was read immediately after mixing at excitation and emission wavelengths of 390 and 470 nm, respectively, with both slits at 5 nm. The hydrophobicity index for each sample was obtained as the slope of the relative fluorescence (difference) between ANS treated and untreated samples versus protein concentration.

Statistical Analysis and Response Surface Methodology Design

Initial preliminary single factor experiments were carried out at pH 5.5. A response surface methodology design (Box-Behnken) was then used to describe the variation of residual inhibitory activity with heat treatment as affected by temperature, heating time and pH. Tests were done triplicates and the design run twice. The experimental design and statistical analysis was done using Design-Expert software version 7.1.5 (Minneapolis, USA). The region of study selected and resultant runs are shown in Table 1 and 2, respectively. Tests for effects of salts and polyols were assessed using two-way ANOVA tests.

RESULTS AND DISCUSSION

Purification and Yield

The SDS-PAGE pattern for the purified inhibitor and the DEAE-Sepharose CL-6B ion exchange chromatogram purification are shown Fig. 1a and b, respectively. According to Le Berre-Anton *et al*. (1997), Pueyo *et al*. (1993) and Moreno *et al*. (1990), the purified amylase inhibitor consists of bands in the range 14400-21000 Da. These bands were suggested to results from denaturation of the pure

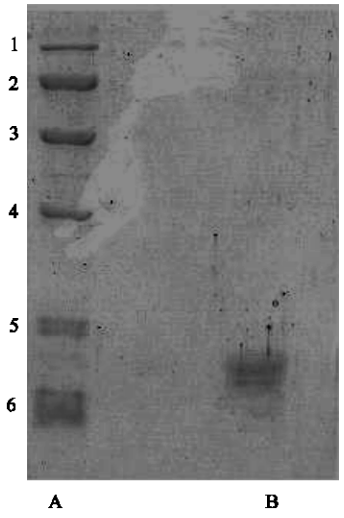

Fig. 1a: SDS-PAGE pattern of purified speckled kidney bean *Phaseolus vulgaris* α-amylase inhibitor (α-AI1). A: Standard markers consisting of 1-Rabbit phosphorylase b (97400 Da), 2-bovine serum albumin (66200 Da), 3-rabbit actin (43000 Da), 4-Bovin carbonic anhydrase (31000 Da), 5-soybean trypsin inhibitor (20100 Da) and 6-Hen egg white lysozyme (14100 Da). B: Purified *Phaseolus vulgaris* alpha-amylase inhibitor (α-AI1)

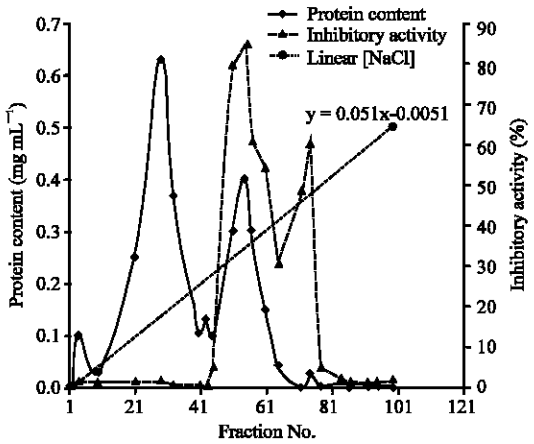

Fig. 1b: DEAE-Sepharose CL-6B Ion exchange chromatogram for purification of *Phaseolus vulgaris* α-amylase inhibitor from speckled kidney beans. Conditions: Flow rate 2 mL min^{-1}, volume per tube 10 mL, elution conditions 0-5 m NaCl in 20 mM Tris HCl buffer pH 7.1, Column dimensions; Diameter = 3 cm, height =19 cm. Collection rate per tube , 10 mL

amylase inhibitor of molecular weight 36 kDa into smaller fractions due to the SDS method (Le Berre-Anton *et al*., 1997). After purification, the inhibitor showed two fused broad bands (Fig. 1a) in the region 14400-21000 Da. The fused bands probably corresponded to the two inhibitor subunits isolated by Yamaguchi (1991). There was no detectable hemagglutinating activity in the purified inhibitor.

Le Berre-Anton *et al*. (1997), Tormo *et al*. (2004) and Marshall and Lauda (1975) reported extraction percentage values of 0.04-0.05, 0.15-0.24 (w/w) and 0.05% (w/w), respectively. The extraction procedure in this study gave an extraction percentage of 0.09% (w/w) of seed flour based on protein content, which was within the range previously reported.

Model and Residual Inhibitory Activity Variation

The resultant model obtained was expressed by Eq. 2 below. The R-Squared and adjusted R-Squared values obtained of 0.9997 and 0.9991, respectively indicated that the model obtained, explained over 99% of the residual α-amylase inhibitory activity variation. A Lack of Fit probability of 0.1939 was obtained which illustrated that it was not relevant relative to the pure error in the model hence the model could be used to predict values given there was only 19.39% chance that the Lack of Fit F-value resulted from noise. The Adequacy of Precision value, which is a measure of the ratio of the signal to noise, was 97.069 compared to the minimum requirement of 4 according to the design.

$$\begin{aligned}\text{Residual inhibitory activity}(\%) = {} & 0.39278A^2C - 1.115367A^2 - 0.070961B^2 - \\ & 0.27061C^2 - 0.064474B + 3.96313AC - 8.87121x \\ & 10^{-3}BC + 23.76840A + 8.21374B - 1.22720C - 251.48409\end{aligned} \quad (2)$$

The variables pH, temperature, (pH-temperature), (pH-time), $(pH)^2$, $(temperature)^2$, $(time)^2$, $(pH)^2$ (time) were found significant. The variables pH (A), Time (C), pH-Temperature (AB) and Temperature-Time (BC) had probability >F values of 0.0056, 0.159, 0.171 and 0.0655, respectively, with the remaining having less than 0.0001. The coefficients showed that the linear terms of pH and temperature had a positive effect on the residual inhibitory activity, while all the interaction terms had negative effects on the residual activity (Eq. 2). The interactive influence of the heating temperature, time and pH on the retention of inhibitory activity are illustrated in Fig. 2a-c.

On heating the inhibitor from 60-90°C (Fig. 2a), the residual activity decreased to <25% for high (7.5) and low pH (3.5), while <44.66% was retained at medium pH (5.5). In addition, the inhibitor showed maximum stability at pH 6.02. This indicated that the inhibitor would be relatively more stable in medium pH food systems than in low (3.5) like some fruit citrus juices or high pH foods. At constant temperature (Fig. 2b) for all pH levels the activity initially increased on heating then decreased, for example at pH 5.5 the activity increased rapidly to a max >83.336 in 10-20 min then decrease thereafter. Therefore, application of the inhibitor before heat treatment, maybe feasible in system like pasteurisation, though it would be significantly and interactively modulated by the food system pH, temperature and heating time. These results were consistent in terms of the stability temperature range with those of Collins *et al*. (2006) and Yoshikawa *et al*. (2000).

Molecular Changes Involved in Activity Loss

Intrinsic flourescence and ANS aided flourescence have been used as versatile tools in many studies to assess changes in food protein structures with denaturing treatments. The fluorescence spectra showing the variation of emission intensity with heating time are shown in Fig. 3a. The spectra showed an increase in emission intensity and no significant shift in the spectra. There was a minor difference in the spectra and maximum intensity between 0, 5 and 15 min of heating while a large increase in the intensity was observed between 15 and 25 min of heating. The difference between 25 and 45 min of heating was less pronounced. The relatively large increase in the tyrosine/tryptophan flourescence between 15 and 25 min indicated a change in the tertiary structure of the inhibitor which accompanied loss of activity. Results from UV-absorbance scan measurements, did not show any peak within the range 250-300 nm. This could have been due to the low tyrosine and tryptophan content of the *Phaseolus vulgaris* alpha-amylase inhibitor as shown by Sawada *et al*. (2001) and Yang *et al*. (2008).

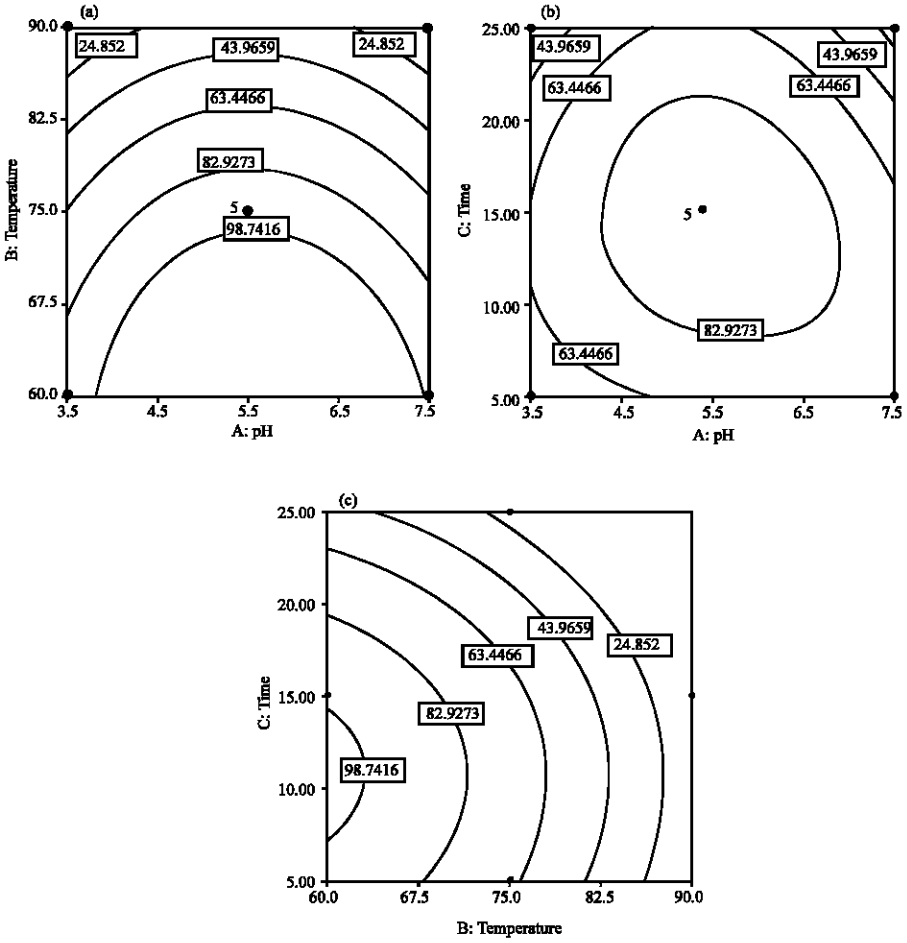

Fig. 2: Contours showing influence of (a) temperature-pH, (b) time-temperature and (c) pH-time interaction on residual *Phaseolus vulgaris* α-amylase inhibitor (α-AI1) inhibitory activity

The ANS flourescence probe is used to amplify changes in the surface hydrophobicity (Ho) of proteins. In the present study, the hydrophilicity index increased with heating time (Fig. 3b). The index at 15 min (H_0 = 168.49; R^2 = 0.96) of heating was relatively close to the initial value (H_0 = 107.63; R^2 = 0.89) and significantly different from that at 25 min (H_0 = 306.7; R^2 = 0.99), while that at 25 and 45 min (H_0 = 311.4; R^2 = 0.96) was only slightly different. A similar trend was observed during the assessment of tranglutaminase heat stability by Cui *et al*. (2008) . The large difference between 15 and 25 min here, indicated that the activity loss was accompanied by the unfolding of the protein through the exposure of hydrophobic residues from the interior to the surface.

Effect of Selected Hofmeister Series Salts and Polyols

Various salts and polyols have been reported to have a significant influence on the activity of bioactive proteins based on their position in the Hofmeister series and the applied concentration (Chi *et al*., 2003; Plaza *et al*., 2008). Figure 4 shows the effect of selected Hofmeister series salts with

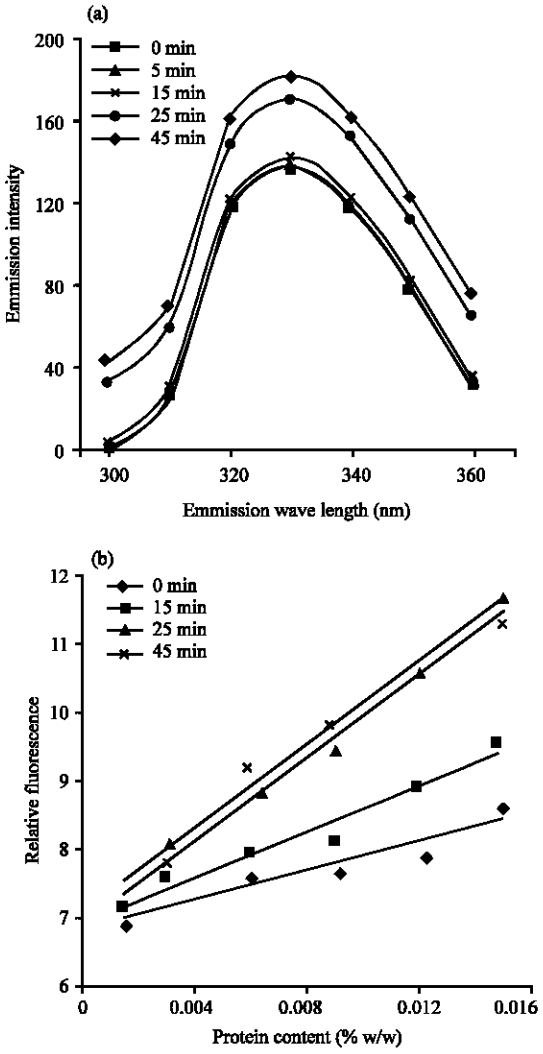

Fig. 3: Effect of heating time on the tyrosine/tryptophan fluorescence (a) and surface hydrophobicity index (b) of the purified *Phaseolus vulgaris* α-amylase inhibitor (α-AI1)

increasing salting-in (kosmotrophic) effect. At high concentration, the protective effect decreased with increasing salting-in-effect from more chaotrophic salts in the order $CH_3COO^- > Cl^- > Br^- > I^- > SCN^-$.

At low concentration the protective effect was relatively low and consistent, but increased sharply with the high salting-in effect (kosmotrophic) salts ($SCN^- > I^-$). The results correlate with the recent reviews by Yamasaki *et al.* (1991), which showed that BSA was stabilised by kosmotrophic salts at low concentration. Such stability was proposed by Chi *et al.* (2003) to be modulated by charge shielding which reduces electrostatic interactions at low concentrations while preferential binding was proposed to be the main modulating effect at high concentration. It can be concluded therefore, that the charge binding effect of more chaotrophic salts has a stabilizing effect of the *Phaseolus vulgaris* alpha-amylase inhibitor while at low concentration, the charge shielding effect of highly kosmotrophic

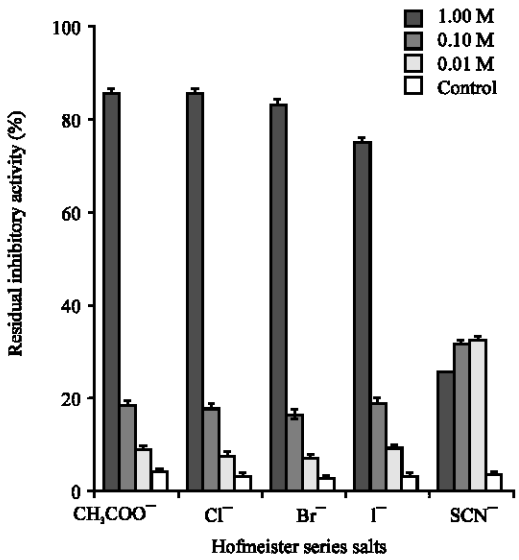

Fig. 4: Effect of selected Hofmeister series salts on the stability of the *Phaseolus vulgaris* α-amylase inhibitor (α-AI1)

effect salts has the same effect. The polyols tested (ethylene glycol, glycerol, sorbitol and polyethylene glycol-2000), at all concentrations (10, 1 and 0.1% w/w), did not have any significant ($p = 0.05$) protective effect on the inhibitor (data not presented).

CONCLUSION

Although application of the *Phaseolus vulgaris* α-amylase inhibitor (α-AI1) before heat treatment is convenient, it may expose it to denaturation. It has been demonstrated in this study that the inhibitor is relatively stable under several heating conditions. The temperature, time, pH and additives like salts in a given food system will however significantly and interactively influence the stability of the inhibitor. If applied before heat treatment in systems like pasteurization, it may therefore provide a convenient agent against diabetes, obesity and related conditions

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