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Chymotryptic hydrolysates of α-kafirin, the storage protein of sorghum (*Sorghum bicolor*) exhibited angiotensin converting enzyme inhibitory activity

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Abstract

Kafirin is the main storage protein (prolamin) in sorghum grains. α-Kafirin, the alcohol soluble fraction, was isolated from sorghum flour. Treatment of α-kafirin with chymotrypsin yielded a hydrolysate which on fractionation, using Sephadex G-25 column, yielded four fractions with significant angiotensin converting enzyme (ACE) inhibitory activity in vitro. The IC_{50} values of these fractions ranged from 1.3 to 24.3 μg/ml. Two of the fractions were found to be competitively inhibiting the enzyme, while two other fractions were non-competitive inhibitors. These results demonstrate that chymotryptic hydrolysates of sorghum prolamin could serve as a good source of peptides with angiotensin I converting enzyme inhibitory activity.

Keywords: Sorghum flour; Angiotensin converting enzyme; Kafirin; Chymotryptic hydrolysate; IC_{50}; Lineweaver–Burk plots

1. Introduction

Angiotensin I converting enzyme (ACE, dipeptidyl carboxy peptidase, EC 3.4.15.1) is a multifunctional zinc-containing enzyme, located in different tissues. This enzyme plays a key physiological role in the control of blood pressure, by virtue of the rennin–angiotensin system (Fujita, Yokoyama, & Yoshikawa, 2000; Ondetti, Rubin, & Cushman, 1982; Rencland & Lithell, 1994). ACE converts the inactive decapeptide, angiotensin I to the potent vasopressor octapeptide, angiotensin II and inactivates bradykinin (Ondetti et al., 1982).

Inhibition of ACE is considered to be a useful therapeutic approach in the treatment of high blood pressure. Besides, several ACE inhibitors may also have beneficial effects on glucose and lipid metabolism (Pollare, Lithell, & Berne, 1989). Several effective oral ACE inhibitors have been developed, namely, captopril, enalapril, and lasinopril and all are currently used as clinical antihypertensive drugs (Ondetti et al., 1982). Although synthetic ACE inhibitors are effective as antihypertensive drugs, they cause adverse side effects such as coughing, allergic reactions, taste disturbances, and skin rashes. Therefore, research and development to find safer, innovative and economical ACE inhibitors is necessary for the prevention and remedy for hypertension. Several food-derived peptides inhibited ACE (Ariyoshi, 1993), which were hydrolyzed by pepsin, trypsin or chymotrypsin including casein (Maruyama et al., 1987), zein (Miyoshi et al., 1991), gelatin (Chen, Ken, & Chang, 1999), yam dioscorin (Hsu, Lin, Lee, Lin, & Hou, 2002), wheat germ (Matsui, Li, & Osajima, 1999) and chickpea (Yust et al., 2003).

Plant seeds, especially cereal seeds are one of the most important sources of proteins (Anantharaman & Finot, 1993) and plant seed storage proteins (prolamins) contain bioactive fragments. The bioactivity of several proline-rich seed proteins have been extensively reviewed (Ariyoshi,

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1993) and the prolamins analyzed have been found to be potential precursors of antihypertensive peptides. Further, it has been reported that oral administration of enzymatic hydrolysates of α-zein induced a decrease in rat blood pressure (Miyoshi et al., 1991). Interestingly, it was observed that antihypertensive peptides from α-zein (proline containing) were not susceptible to proteolysis by enzymes of the digestive tract such as chymotrypsin, trypsin or pepsin (Dziuba, Minkiewicz, Puszka, & Dabrowski, 1995).

Sorghum is an important food for people living in the semi-arid tropical areas of Africa and Asia (Murthy & Kumar, 1995). Sorghum flour is rich in phytochemicals with a potential to impact human health in a beneficial manner (Awika & Rooney, 2004; Kamath, Chandrashekar, & Rajini, 2004). The storage proteins of sorghum (kafirins) constitute 50–60% of the total protein of the grain (Paulis & Wall, 1979) and have been classified into three main groups, according to their molecular weight, extractability and structure (Shull, Watterson, & Krleis, 1991). The sorghum kafirin is reported to reveal extensive homology with zein, the major storage protein from maize (De Rose et al., 1988). Studies of Miyoshi et al. (1991) have clearly demonstrated the potent ACE-inhibitory activity of α-zein hydrolysate. However, sorghum storage proteins have not been studied for their potential to yield ACE inhibitory peptides. In this work we report for the first time that the proteolytic hydrolysates of α-kafirin possess ACE inhibitory activities. We also discuss the inhibitory mechanism of the hydrolytic fractions based on Lineweaver–Burk plots.

2. Materials and methods

2.1. Materials and chemicals

Sorghum (*Sorghum bicolor*) a local white variety (M-35-1) used in this experiment was obtained from a local market in Mysore, India. ACE was extracted from pig lung acetone powder (Okamoto, Hanagata, Kawamura, & Yanagida, 1995) and its activity was determined by using Hippuryl-histidyl-leucine (HHL-Sigma chemical Co., St. Louis, MO, USA) as a substrate. Captopril was purchased from ICN (M/s ICN Biomedicals Inc., Aurora, Ohio, USA.) Chymotrypsin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sephadex G-25 was procured from Pharmacia Biotech AB (Uppsala, Sweden). Unless otherwise specified, all chemicals and solvents were of analytical grade.

2.2. Extraction of sorghum prolamins (α-kafirin)

Sorghum grains were ground to flour using a 'Disk Mill' (Glen Mills Inc., Clifton, NJ, USA) to whole grain flour through a 0.5-mm mesh screen. The flour was then defatted by shaking with petroleum ether for 6 h at room temperature. The defatted flour was desolventised in an oven at 40 °C for 4 h. α-Prolamins were extracted from the defatted flour by the method of Mazhar, Chandrashekar, and Shetty (1993). Samples (100 g) were extracted for 6 h on a shaker at 37 °C with *tert*-butanol (1:5, flour: solvent). The suspension was centrifuged at 5000*g* for 15 min and the supernatant was saved. The procedure was repeated twice and the supernatants pooled, and then lyophilized. The α-kafirin thus obtained was checked for homogeneity by SDS–PAGE.

2.3. Chymotryptic hydrolysis of α-kafirin

The lyophilized α-kafirin was dissolved (5mg/ml) in Tris–HCl buffer (200 mM, pH 7.6) containing SDS (2%). Chymotrypsin was added to the α-kafirin solution (40 μg/mg α-kafirin) and the mixture was hydrolyzed at 37 °C for 4 h. The reaction was stopped by heating in a boiling water bath for 5 min and the hydrolyzate was centrifuged for 20 min at 4 °C and 3000*g*. The supernatant was used as the digest.

2.4. Sephadex G-25 gel filtration

A sample of the supernatant was concentrated 5-fold with a vacuum concentrator, and 0.5 ml of the concentrate (1mg protein) was applied to a Sephadex G-25 column (2 cm × 18 cm) equilibrated with water. Water was used as the eluent at a flow rate of 1 ml/min. Fifty fractions (2 ml each) were collected and the absorbance was monitored at 220 nm and the protein content determined (Lowry, Rosebrough, Farr, & Randall, 1951). All the fractions were then screened for ACE inhibitory activity as detailed below.

2.5. ACE inhibitory assay

The ACE inhibitory activity was measured spectrophotometrically using HHL as the substrate, using a modification of the method of Cushman and Cheung (1971). The assay was conducted in a Tris buffer (125 mM, pH 8.3) containing 300 mM NaCl. HHL (25 mM) and an appropriate quantity of the hydrolysate fraction were mixed with the buffer and incubated with ACE (4 mU) at 37 °C for 60 min. The concentration of ACE inhibitors needed to inhibit 50% of ACE activity was defined as the IC_{50} value. Captopril ($IC_{50} = 0.0077$ μM) was used as positive control for ACE inhibition (Hsu et al., 2002).

2.6. Determination of the kinetic properties of ACE inhibition by the fractions

To investigate the inhibition pattern on ACE, the kinetic properties of the enzyme (4 mU) without or with the fractions were determined using different concentrations of HHL (16.7–83.9 μM). The K_m (without the fractions) was calculated from Lineveweaver–Burk plots and the K_i (with fractions) was calculated using the equation $K_i = [I]/(K_{m'}/K_m) - 1$ where [I] is the concentration of the inhibitor

added and $K_{m'}$ is the Michaelis constant in the presence of inhibitor at concentration [I].

3. Results

3.1. Hydrolysis and fractionation of α-kafirins

The yield of α-kafirins was 0.05% (w/w). SDS–PAGE revealed a predominant band around 27 kDa and a faint band around 17 kDa (Fig. 1). Incubation of α-kafirin with chymotrypsin yielded complete hydrolysis, which was confirmed by SDS–PAGE (data not shown). The digest on fractionation through Sephadex G-25 column yielded fractions with protein content as shown in Fig. 2.

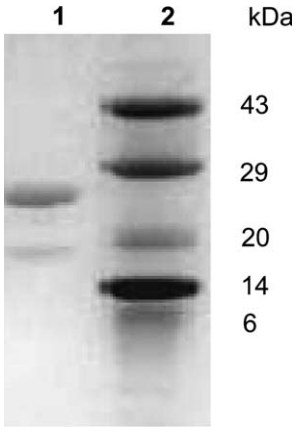

Fig. 1. SDS–PAGE of: α-kafirin from sorghum (lane 1); molecular weight marker (lane 2).

3.2. ACE inhibitory activity of the fractions

Several of the fractions exhibited ACE inhibitory activity (7–46% inhibition) under the experimental conditions (Fig. 2). Specifically, fractions 25, 26, 33 and 38 inhibited ACE by 35%, 46%, 30% and 35%, respectively, at equal volumes (50 μl). Hence these fractions were employed for further studies. These fractions exhibited concentration-dependent ACE inhibitory activities and the 50% inhibition of ACE activity (IC_{50}) was determined to be 24.3, 9.3, 4.0 and 1.3 μg/ml for fractions 25, 26, 33 and 38, respectively, compared to that of 0.0077 μM, 1.7 ng/ml for captopril (Table 1).

3.3. Kinetic properties of ACE inhibition by the fractions

The kinetic parameters were calculated using Michaelis–Menten equation and are presented in Table 2. The Lineweaver–Burk plots of ACE (4 mU) without or with selected fractions in different concentrations of HHL are presented in Figs. 3 and 4. The results indicate that the fractions examined acted as competitive (fractions 25 and 26; Fig. 3(a) and (b)) or uncompetitive (fractions 33 and 38; Fig. 4(a) and (b)) inhibitors with respect to the substrate (HHL). The calculated K_i value for the fraction

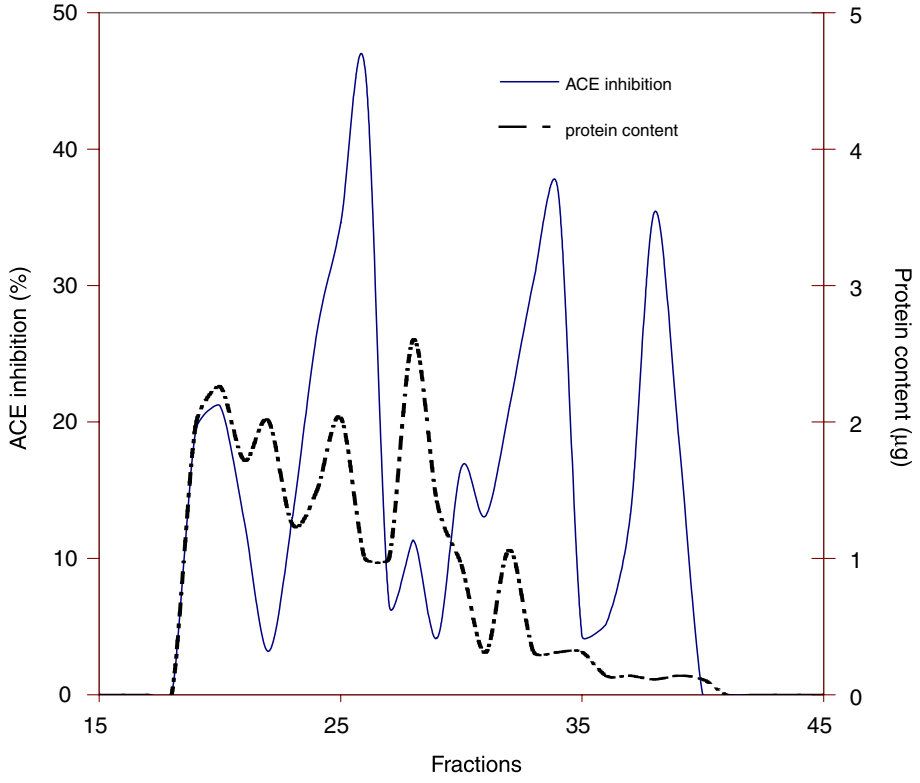

Fig. 2. ACE inhibitory activity of α-kafirin hydrolysates: Sephadex G-25 fractions of chymotryptic digest showing ACE inhibition vs. protein content.

Table 1
IC_{50} values and comparative yield of the different fractions of α-kafirin hydrolysate

Fractions	IC_{50} (μg/ml)[a]	Yield[b] (%)
25	24.3	9.3
26	9.3	4.6
33	4.0	1.4
38	1.3	0.5

[a] Concentration of an inhibitor required to inhibit 50% of ACE activity; IC_{50} calculated plotting means of three independent determinations.
[b] Percentage of protein in relation to the total protein loaded on to the column.

Table 2
Kinetic parameters of ACE in the presence of selected hydrolytic fractions

Parameters	Fraction #			
	25	26	33	38
$1/V_{max}$	0.0023	0.0023	0.0034	0.0027
K_m/V_{max}	0.2133	0.1978	0.1827	0.1959
V_{max} (nm)	434.78	434.78	294.11	370.3
K_m (μM)	92.73	86.00	53.73	72.55
$1/K_m$	0.0107	0.0116	0.0186	0.0137
K_i (μg)	11.23	4.47	2.38	0.68

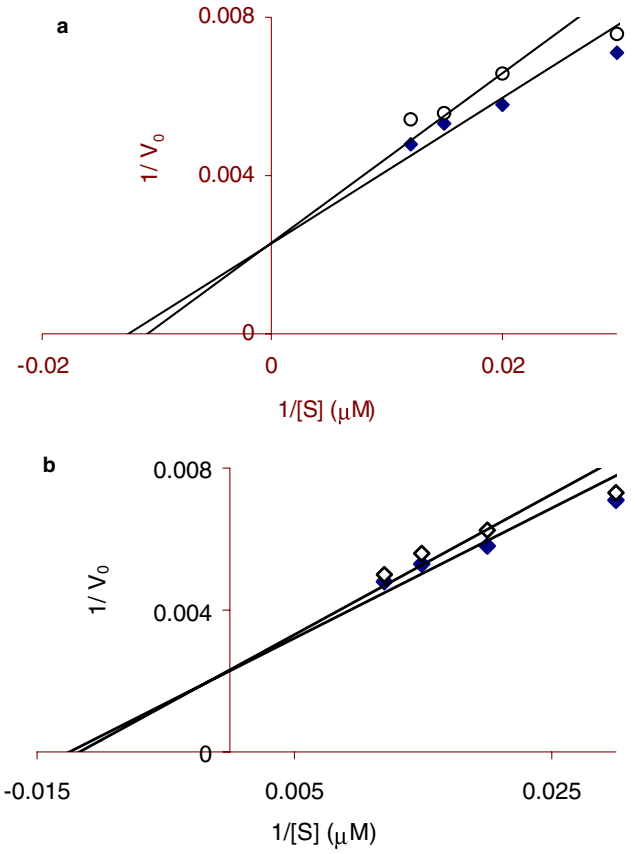

Fig. 3. Lineweaver–Burk plots of inhibition of ACE by the selected Sephadex G-25 fractions of chymotryptic hydrolysates of sorghum α-kafirin. Each point represents the mean value of three experiments. ACE activities were measured in the absence (◆) or in the presence of the hydrolytic fractions at their IC_{50} concentration: (a) fraction 25 (○); (b) fraction 26 (◇).

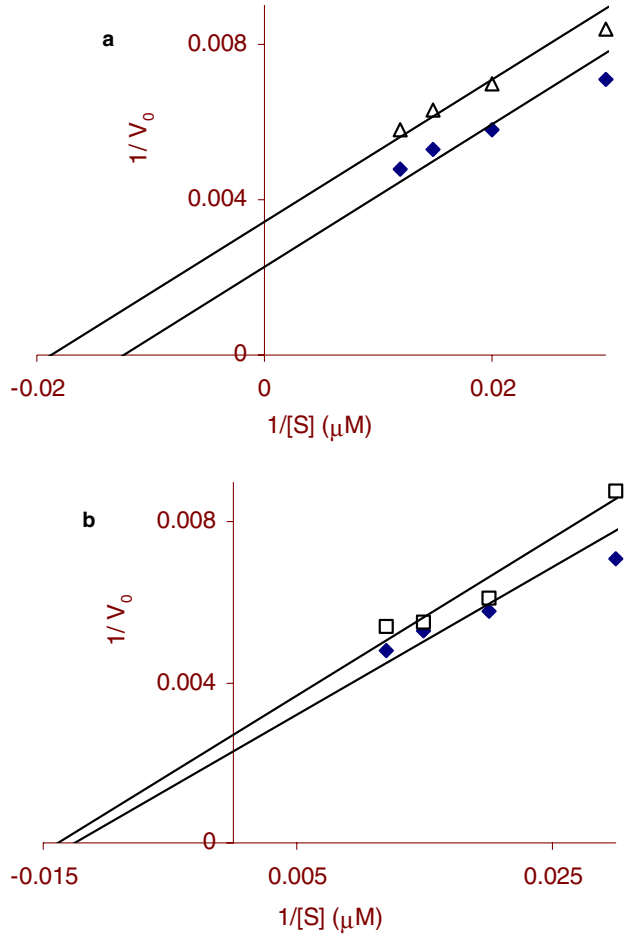

Fig. 4. Lineweaver–Burk plots of inhibition of ACE by the selected Sephadex G-25 fractions of chymotryptic hydrolysates of sorghum α-kafirin. Each point represents the mean value of three experiments. ACE activities were measured in the absence (◆) or in the presence of the hydrolytic fractions at their IC_{50} concentration: (a) fraction 33 (△); (b) fraction 38 (□).

was No. 25, 11.23 μg; No. 26, 4.47 μg; No. 33, 2.38 μg and No. 38, 0.68 μg for HHL.

4. Discussion

Several bioactive peptides have been isolated from enzymatic hydrolysates of food proteins (Maruyama et al., 1987; Mullally, Meisel, & FitzGerald, 1996). Especially, plant seed storage proteins are reported to contain bioactive fragments (Dziuba et al., 1995), which may be liberated by proteolytic enzymes. Further, proline-rich proteins or protein fragments are speculated to be precursors of antihypertensive peptides. Such bioactivity of known-proline-rich food proteins were reviewed by Ariyoshi (1993) and all prolamins analyzed were found to be potential precursors of antihypertensive peptides. However, only a few kinds of prolamins such as α-zein (Miyoshi et al., 1991) and γ-zein (Maruyama, Miyoshi, Kaneko, & Takana, 1989) have been employed for obtaining ACE inhibitory peptides. Kafirins, the prolamin storage protein of sorghum grains is reported

to be of poor quality nutritionally since it contains practically no lysine and little tryptophan and threonine (Guiragossian et al., 1978). Sorghum kafirin is reported to have proline rich fragments (De Rose et al., 1988) and hence the present study was undertaken to exploit its utility as a source of antihypertensive fractions.

In the present study, α-kafirin on hydrolysis with chymotrypsin yielded a hydrolysate, which on gel filtration yielded four fractions possessing significant ACE inhibitory activity. The ACE inhibitory activity of these fractions was higher than that reported for the hydrolysates of certain other cereals and legumes (Rhyu, Nam, & Lee, 1996). The kinetic analysis of the results suggests that two of the fractions inhibit the enzyme activity by competitively binding with the substrate for the active sites, while two other fractions were uncompetitive inhibitors.

It was earlier reported that tandem repeated proline-rich sequence of maize endosperm proteins possessed strong ACE inhibitory activities (Maruyama et al., 1989). Chymotryptic hydrolysate of α-zein has been shown to possess ACE inhibitory activity (Miyoshi et al., 1991). Earlier reports indicate that the consensus repeat motifs/sequences features of α-zein of maize and α-kafirin of sorghum are identical (Chandrahsekar & Mazhar, 1999). Hence we presume that the peptides in α-kafirin hydrolysates in the present study may have similar peptide sequence and hence possess strong ACE inhibitory activity in vitro.

In conclusion, hydrolysis of sorghum kafirin using the protease chymotrypsin yielded a hydrolysate rich in peptides with ACE inhibitory activity. The hydrolysates alternatively could be used as a starting material for antihypertensive drugs as ACE inhibitors. Isolation of specific peptides from these fractions can be achieved by reverse phase chromatography. It remains to be tested whether these peptides may exert antihypertensive activity in vivo. In order to have activity in vivo, the peptides would have to be resistant to endogenous digestive enzymes so that they could reach the blood stream intact. Further, it would be interesting to know whether these fractions would be released by endogenous chymotryptic hydrolysis in vivo to inhibit ACE as part of the physiological regulation.

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