Intl. J. of Food. Ferment. Technol. 3(1): 71-77, June, 2013

DOI Number: 10.5958/j.2277-9396.3.1.007

Angiotensin-I Converting Enzyme (ACE) Inhibitory Activity of Fermented *idli* Batter as Influenced by Various Parameters Prevailing During Fermentation

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Paper no: 63 **Received**: 11 February , 2013 **Received in revised form**: 19 May , 2013 **Accepted**: 21 June, 2013

Abstract

Angiotensin-I converting enzyme (ACE) inhibitory peptides known for regulating blood pressure are formed in foods from proteins due to hydrolysis occurring *in vitro* in processes such as fermentation as well as *in vivo* due to digestion in the gut. *Idli* is a cereal-legume based fermented snack food widely consumed in southern India. In the present work ACE inhibitor potential of fermented *idli* batter was estimated for the first time. Various factors prevailing during *idli* batter fermentation such as time of fermentation, proportion of rice and black gram dhal used in preparation of batter, temperature of fermentation, addition of an extrinsic protease and refrigerated storage of batter were found to have an effect on ACE inhibition. On addition of 344 U of extrinsic protease per gram of batter, IC_{50} value was reduced by more than tenfold to 0.480 mg of protein/ml signifying the formation of more and/ or more potent ACE inhibitory peptides.

Keywords: ACE inhibitor, *Idli* batter, IC50 value, Fermentation, Protease addition

Introduction

Hypertension is one of the major independent risk factors for arteriosclerosis, stroke and myocardial infarction. Angiotensin-I converting enzyme (ACE; EC 3.4.15.1) associated with regulation of blood pressure; is a dipeptidyl carboxypeptidase located primarily on the luminal surface of vascular endothelial cells (especially in lungs) (Tripathi, 2008). It increases blood pressure by two ways: firstly by converting inactive decapeptide angiotensin-I to an active octapeptide angiotensin-II (a potent vasoconstrictor); secondly by inactivating the vasodilator bradykinin. ACE inhibitor drugs prevent the formation of angiotensin-II which is responsible for constriction of blood vessels and thereby lowers the blood pressure (Sweitzer, 2003). The major side effects of synthetic ACE inhibitor drugs are cough, skin rashes and allergic reactions. This explains the need for developing safer and natural ACE inhibitors.

ACE inhibitors obtained from daily dietary food proteins serve as a novel approach for nutrition. Many bioactive peptides from enzymatic hydrolysate of cowpea (Campos *et al*., 2011), defatted soybean flour (Gouda *et al*., 2006), defatted canola meal (Wu *et al*., 2008), corn gluten (Suh *et al*., 2003), buckwheat flour (Ma *et al*., 2006) have been found to inhibit ACE; both *in vivo* and *in vitro*. Since ancient times, fermented foods form an important part of diet.

During the process of fermentation, microbial induced proteolysis can help in formation of bioactive peptides. Many fermented foods such as fish sauce, soy sauce, cheese, miso, natto, temphe etc. are found to contain potential ACE inhibitory peptides (Okamoto *et al*., 1995).

Indian diet consists of various fermented foods; *Idli* is one such cereal-legume based fermented food. The major ingredients are parboiled rice (*Oryza sativa*) and black gram dhal (*Phaseolus mungo*) which are soaked and ground separately. This is followed by mixing of the two batters with addition of small amount of salt. This is then allowed to ferment overnight at room temperature. The fermented batter is steamed in special *idli* pans for 5-8 minutes (Nagaraju and Manohar, 2000).

This is the first ever approach to explore Indian traditional fermented food system as a source of ACE inhibitory peptides. Herein we report the estimation of ACE inhibition from fermented *idli* batter. Moreover, the effect of various parameters during *idli* batter fermentation such as fermentation time, rice and dhal proportion, fermentation temperature, refrigerated storage and extrinsic protease addition on ACE inhibition has been investigated.

Materials and Methods

Materials and Chemicals

Parboiled rice and black gram dhal were procured from local market. Rabbit lung acetone powder as source of ACE, captopril, hippuryl-L-histidyl-L-leucine (HHL) and hippuric acid (HA) were purchased from Sigma-Aldrich, India. Pyridine and benzene sulfonyl chloride (BSC) were obtained from Merck, India. The fungal protease (*Aspergillus* spp.) was obtained as a gift sample from Advance Enzymes Ltd., India. All other reagents and chemicals used were of analytical grade.

Preparation of *Idli* batter

The raw material (three parts of parboiled rice and one part of black gram dhal) were soaked separately in water at room temperature (27±2°C) for 4 h. These were then ground separately in a grinder, with black gram dhal being ground to a fine paste and rice ground to a coarse consistency. The two batters were then mixed and 0.9% w/w salt was added. The batter was allowed to ferment for 14 h at room temperature. This batter is denoted as batter 'S'. In the present work, this method for preparation of batter was followed for all experiments unless otherwise specified. For all studies three sets of batter were prepared. The data reported is mean ± s.d. of triplicate determinations of three sets of batter.

Preparation of ACE inhibitory extract from *Idli* batter

The extract was prepared by modified method of Kuba *et al*., (2003). The *idli* batter was centrifuged at 10,000 rpm for 45 min at 4°C and the resulting pellet was discarded. The supernatant obtained was boiled for 20 min followed by centrifugation at 10,000 rpm for 45 min at 4°C. The pH of supernatant was adjusted to 8.2 with 1 N NaOH and was again centrifuged at above mentioned conditions. This supernatant served as ACE inhibitory extract and was stored at 4°C till further use.

Protein Estimation

The protein concentration was determined by the method of Lowry *et al*., (1951). Bovine serum albumin was used as the standard.

Determination of the ACE inhibition activity

The ACE inhibitory activity was assayed by using the modified method of Jimsheena and Gowda (2009). ACE hydrolyzes HHL to hippuric acid and *histidyl-leucine*. This method relies on colorimetric reaction of hippuric acid with pyridine and BSC as reported by Umberger and Fiorese (1963). For each assay, 25 µl of rabbit lung ACE enzyme extract was preincubated with 25 µl of *idli* batter extract solution at 37°C for 15 min. The reaction was further continued by addition of 50 µl of 5 mM HHL and 100 µl of 0.05 M sodium borate buffer (pH 8.2) containing 300 mM NaCl for 30 min. The reaction was stopped by adding 200 µl of 1 M HCl. The liberated HA was measured by complexing it with 400 µl of pyridine and 200 µl of BSC which forms a yellow color and is measured at 410 nm. Percentage ACE inhibition was calculated using below mentioned formula:

$$\text{ACE inhibition (\%)} = \{(A_a - A_p) / A_a\} * 100$$

where, A_a: ACE activity in the absence of inhibitor**;** A_p: ACE activity in the presence of inhibitor. The IC_{50} value was defined as the concentration of *idli* batter extract required to decrease the ACE activity by 50%.

Effect of fermentation time on ACE inhibitory activity of *idli* batter extract

The *idli* batter was prepared as mentioned previously i.e. **batter 'S'**. The prepared *idli* batter was divided into 9

parts having equal weight. One of them was used as control i.e. it was not fermented (0 h). The other 8 sets of batter were fermented at room temperature (27±2°C) for 10, 12, 14, 16, 18, 20, 22 and 24 h respectively. ACE inhibitory extract was prepared from these batters and evaluated for percentage ACE inhibition by the method mentioned previously.

Effect of rice and dhal proportion on ACE inhibitory activity of *idli* batter extract

For 2:1 proportion two parts of rice and one part of dhal, for 3:1 proportion three parts of rice and one part of dhal and for 4:1 proportion four parts of rice and one part of dhal was used and rest of the procedure for preparation of batter remained same as mentioned previously. In all the cases the batter was fermented for 14 h at room temperature (27±2°C). ACE inhibitory extract was prepared from these batters and evaluated for percentage ACE inhibition by the method mentioned previously. The batter prepared from 2:1 proportion of rice: dhal and fermented for 14 h at room temperature is denoted as batter 'A'.

Effect of fermentation temperature on ACE inhibitory activity of *idli* batter extract

The *idli* batter was prepared as described previously i.e. batter 'S'. The prepared batter mixture was divided into 4 parts having equal weight. One of them was used as control and was fermented at room temperature (30±2°C). The other 3 sets of batter were fermented at 25±2°C, 37±2°C and 45±2°C respectively. In all cases batter was fermented for constant time period i.e. 14 h. ACE inhibitory extract was prepared from these batters and evaluated for percentage ACE inhibition by the method mentioned previously. The batter prepared from 3:1 proportion of rice and black gram dhal respectively, fermented at 37±2°C for 14 h is denoted as batter 'B'.

Effect of refrigerated storage time on ACE inhibitory activity of *idli* batter extract

The *idli* batter was prepared as mentioned previously i.e. batter 'S'. The batter mixture was fermented for 14 h at room temperature (27±2°C). The fermented batter mixture was then divided into 5 parts having equal weight. One of them was used as control and labeled as Day 1 fermented batter. The other 4 sets of batter were refrigerated at 6±2°C and labeled as Day2, Day3, Day4, and Day5 respectively. ACE inhibitory extract was prepared from these batters and evaluated for percentage ACE inhibition as described earlier.

Preparation of batter for protease addition studies and effect on ACE inhibitory activity of *idli* batter extract

The *idli* batter was prepared as described previously i.e. batter 'S'. The batter mixture was divided into 4 parts having equal weight. One of them was used as the control (0 U of protease) and in other three sets of batter, calculated amounts of fungal protease enzyme having an activity of 25771.34 U/g of solid was added to obtain i.e. 0.6872U, 6.8723U, 343.617U activity per gram of batter respectively. The three batters along with the control batter were allowed to ferment for 14 h at room temperature (27±2°C). ACE inhibitory extract was prepared from these batters and evaluated for percentage ACE inhibition. Also, IC_{50} value of batter containing 343.617 U of added fungal protease per gram of batter was evaluated. The batter prepared with 3:1 proportion of rice and black gram dhal respectively, fermented for 14 h at room temperature and addition of 343.617U protease per gram of batter is denoted as batter 'C'.

Preparation of batter using optimized values of parameters and estimation of ACE inhibitory activity

Idli batter was prepared using 2:1 proportion of rice and black gram dhal. Fungal protease i.e. 343.617 U of enzyme was added per gram of batter at the start of fermentation. It was allowed to ferment for 14 h at 37°C. ACE inhibitory extract was prepared from fermented batter and evaluated for percentage ACE inhibition. The batter so prepared is denoted as batter 'D'.

Results And Discussion

Fig. 1 represents the effect of fermentation time on percentage ACE inhibition. Significant increase in percentage ACE inhibition was observed between 0 and 10 h fermented batter. The increase in ACE inhibition during the process of fermentation of *idli* batter could be due to microbial growth accompanied by increase in protease activity. The released protease breaks down the protein substrate to form peptides. Inhibitory effect remained relatively constant for 12, 14, 16 and 18 h fermented batter. After 18 h there is steady decline in ACE inhibitory activity from 75.275% for 20 h fermented batter to 69.477% for 24 h fermented batter. A likely reason for the observed decline can be due to the progressive hydrolysis of initially formed potent peptides. The results obtained were analyzed

by standard analysis of variance (Single Factor ANOVA) indicating P value to be less than 0.05, hence the analysis was significant. In other words, percentage ACE inhibition for varying time period of fermentation is not same. The reported study on douchi a fermented soybean product, found increase in percentage ACE inhibition from 24 h to 15 days. Also, 72.4% of ACE inhibition was observed after long 72 h of fermentation (Zhang *et al*., 2006). In this study on *idli* batter, considering the 0 h reading the net percent of ACE inhibition i.e. 36.815% was observed merely after 10 h of fermentation which is half of that observed after 72 h of fermentation in douchi. This shows that Indian traditional fermented food system i.e. *Idli* batter though fermented for shorter time (10-14 h) can serve as better source of ACE inhibitory peptides.

Fig. 2 represents the effect of different proportions of rice and black gram dhal on percentage ACE inhibition. Maximum ACE inhibition was observed with 2:1 proportion (batter 'A') as compared to other two proportions. There could be two possible reasons for high ACE inhibitory activity. In *idli* batter prepared with higher amount of black gram dhal ingredient, the total quantity of protein in the batter will increase as dhal has a higher content of protein than rice which may eventually lead to a greater formation of ACE inhibitory peptides on fermentation due to proteolysis. Secondly it is possible that the amino acid sequences in black gram dhal protein may be more favorable towards formation of more potent ACE inhibitory peptides than those present in rice protein. Finally the progress of fermentation may itself be affected by the proportion of dhal present as black gram dhal is thought to majorly contribute to the microbes involved in the fermentation (Neelgund *et al*. 1984). For 3:1 and 4:1 proportion of rice and black gram dhal the percentage ACE inhibition of the *idli* batter extract was found to be significantly less as compared to 2:1 proportion (batter 'A').

Fig. 3 represents the effect of fermentation temperature on percentage ACE inhibition. The results analyzed by standard analysis of variance (Single Factor ANOVA) indicated that there was a significant effect of fermentation temperature on the ACE inhibitory activity ($p < 0.05$). As the fermentation temperature increases from 25°C to 37°C the ACE inhibitory activity also increases. Maximum ACE inhibition was observed when the batter was fermented at 37°C (batter 'B'). The change in percentage ACE inhibition with the change in temperature can be due to varying activity of the enzyme protease contributed by fermenting micro-flora. Enzymes are sensitive to temperature changes up until a certain temperature and will increase in their activity up to this point and further it will decrease. The chief microorganisms involved in *idli* batter fermentation are lactic acid bacteria and yeasts. The optimum temperature in which yeast and lactic acid bacteria enzyme work most efficiently is around 37°C below this the rate of reaction is slow and above 45ºC the enzyme would denature. This could be the reason for maximum ACE inhibition at 37°C (batter 'B').

Fig. 4 represents the effect of refrigerated storage time on percentage ACE inhibition. The results analyzed by standard analysis of variance (Single Factor ANOVA) suggest that there is a significant effect of refrigerated storage time on the ACE inhibitory activity ($p < 0.05$). The difference between percentage ACE inhibition for Day 1 and Day 2 was found to be insignificant ($p > 0.05$) but further storage of batter has affected percentage ACE inhibition. The ACE inhibition was found to decrease sharply for Day 3 and Day 4 indicating further proteolytic modification of active peptides present at Day 1 and Day 2. For 5th Day, i.e. last day of storage ACE inhibition was found to be restored and it was same as Day 1 and Day 2. Due to continued action of enzyme protease, inactive peptides were further hydrolyzed on Day 5 and resultant peptides are likely to be ACE inhibitory, therefore the percentage ACE inhibition was found to be restored on 5th Day. The results obtained in this present study of *idli* batter are in agreement to that of probiotic yogurt (Donkor *et al*., 2007). Probiotic yogurt was refrigerated and ACE inhibitory activity was determined at weekly intervals during 28 days of cold storage at 4°C. Yogurt showed appreciable percentage ACE inhibition during initial stages of storage with significant decrease afterwards. The greatest ACE inhibition was found during 1st and 3rd week of storage.

Fig. 5 represents the effect of addition of extrinsic protease addition on percentage ACE inhibition. The present work on effect of protease addition on percentage ACE inhibition was carried out to understand if in the presence of extrinsic protease extensive hydrolysis of protein moiety of batter takes place which in turn will result in the formation of more than usual number of peptides. During the same 14 h of fermentation control batter (without protease) has shown significantly less percentage of ACE inhibition as compared to batter containing protease. This demonstrates that more number and/or more potent ACE inhibitory peptides are formed because of action of extrinsic protease on the protein content of *idli* batter. The results obtained in this present study on *idli* batter are in an agreement to that on

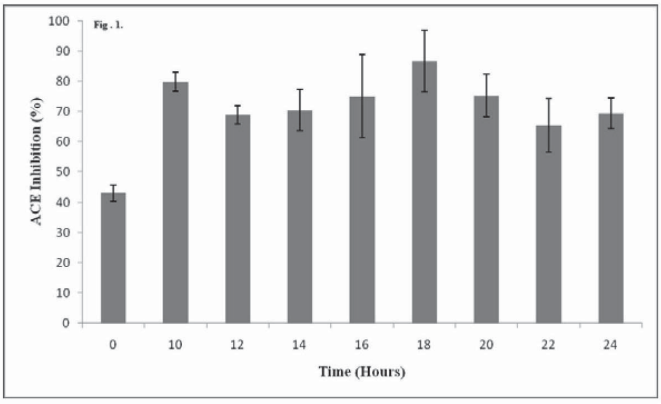

Fig. 1. Percentage ACE inhibition as function of time

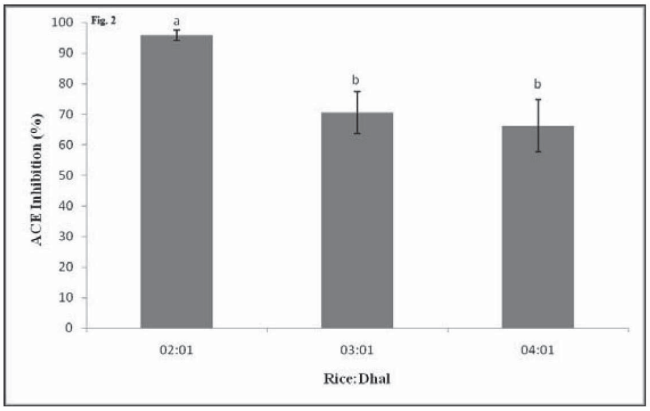

Fig. 2. Percentage ACE inhibition as function of rice and black gram dhal proportion (Means with different letters are significantly different (P<0.05) whereas with same letters are not significantly different (P>0.05) by t-test)

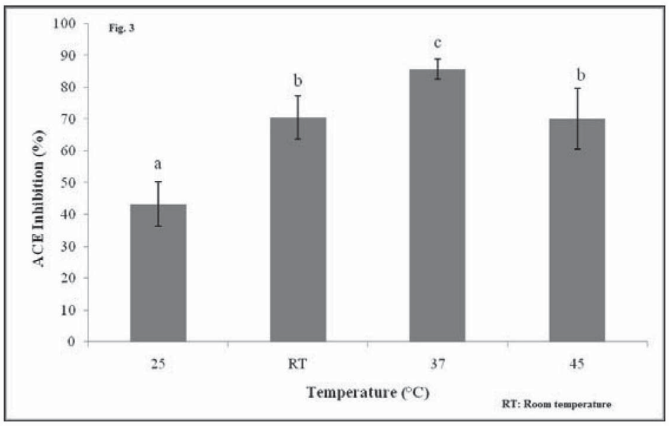

Fig. 3. Percentage ACE inhibition as function of fermentation temperature (Means with different letters are significantly different (P<0.05) whereas with same letters are not significantly different (P>0.05) by t-test)

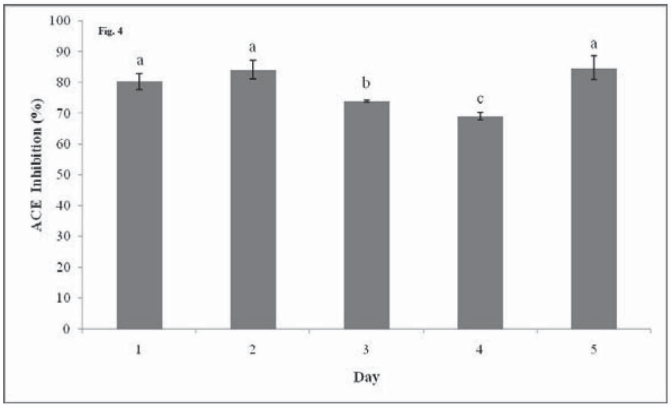

Fig. 4. Percentage ACE inhibition as function of refrigerated storage time (Means with different letters are significantly different (P<0.05) whereas with same letters are not significantly different (P>0.05) by t-test)

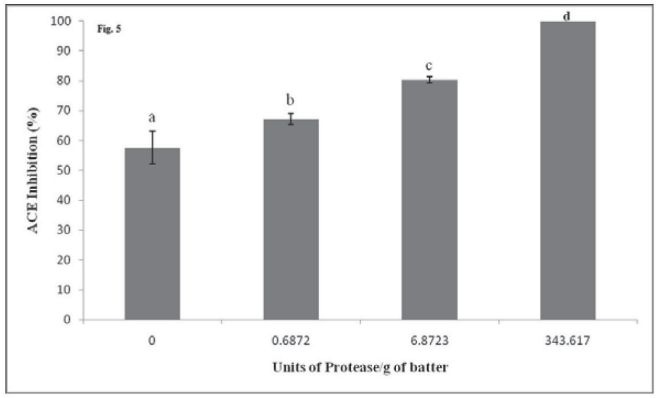

Fig. 5. Percentage ACE inhibition as function of extrinsic protease added (Means with different letters are significantly different (P<0.05) whereas with same letters are not significantly different (P>0.05) by t-test)

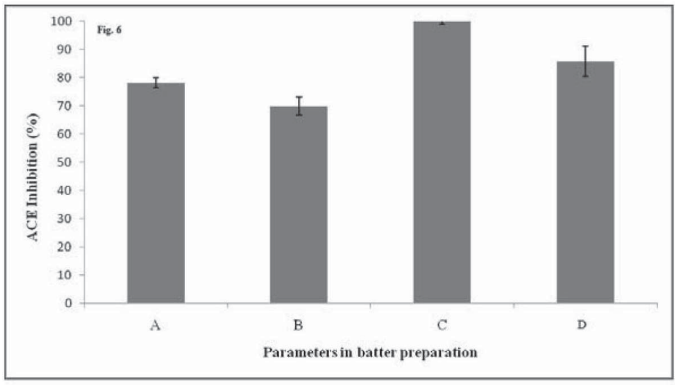

Fig. 6. Percentage ACE inhibition as function of parameter in batter preparation

fermented milk (Phiromruk *et al*., 2009). Milk was fermented with starter cultures namely *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in the presence and absence of protease. 92% of ACE inhibition was obtained merely after 6 h of fermentation in the presence of protease whereas similar amount of inhibition was obtained after 24 h of fermentation in the absence of protease. Hence the added protease was found to be effective in increasing the percent ACE inhibition.

A batter 'D' was prepared combining all the optimized parameters in a single batch viz., 2:1 rice: dhal proportion, fermentation temperature of 37°C, addition of 343.617U of protease per gram of batter and fermentation time of 14 h. Fig. 6 compares the percentage ACE inhibition of the batter so prepared i.e. batter 'D' with that of the other batters prepared under specified conditions (batter 'A', 'B' and 'C'). As observed from the figure, combination of the best conditions in a single batch (batter 'D') has shown greater inhibition than individual conditions of rice and dhal proportion (2:1) (batter 'A') and optimum temperature i.e. 37°C (batter 'B') but has shown lesser inhibition than individual study on protease addition (batter 'C'). The batter prepared by combining the best individual conditions in a single batch (batter 'D') is highly rich in protein content because two parts of rice are used against one part of dhal and further protease has been added. The extent of proteolysis is therefore more in this batter (batter 'D') as compared to that of batter 'C' which is justified by increased Folin-Lowry protein content in combined condition batter 'D' (26.887 mg/ml) as compared to batter 'C' (20.45 mg/ml). As expected the increase in proteolysis should have resulted in increased percentage of ACE inhibition in batter 'D' as compared to batter 'C'. In the present work, the opposing results obtained could be due to formation of inactive ACE inhibitory peptides from those of active ones because of high degree of proteolysis which resulted in further cleavage of active peptides. This might be the reason for the decreased ACE inhibition of the extract obtained from combined parameters in batter preparation (batter 'D') as compared to that of individual study on protease addition (batter 'C'). Thus the ACE inhibitory activity of *idli* batter can be increased by addition of extrinsic protease to a greater extent than by increasing the dhal content in the batter.

Table 1 represents the IC_{50} value of captopril (synthetic ACE inhibitor) and different *idli* batter samples viz., batter 'S', batter 'C' and batter 'D'. In the presence of added extrinsic protease more number and/or more potent ACE inhibitory peptides are formed hence IC_{50} value of batter 'C' is less than one tenth of the value obtained in the absence of extrinsic protease i.e. batter 'S'. The results of this study are on the same lines to that of fermented milk (Tsai *et al*., 2008). Fermentation of milk was carried out using two lactic acid bacteria namely; *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in the presence and absence of extrinsic protease. IC_{50} value of milk fermented for 5 h at 43°C was found to be 0.515 ± 0.012 and 0.266 ± 0.024 mg peptide/ml in the absence and presence of extrinsic protease respectively. IC_{50} value of batter 'D' (4.378 mg of protein/ml) was found to be higher than batter 'C' (0.480 mg of protein/ml) but lower than that of batter 'S' (7.558 mg of protein/ml). The probable reason for being higher than batter 'C' can be due to extensive proteolysis of the formed active peptides to the inactive ones. Thus the ACE inhibitory activity of *idli* batter can be increased by addition of extrinsic protease to a greater extent than by increasing the dhal content in the batter. All IC_{50} values of *idli* batter samples are much higher than the synthetic drug captopril suggesting the lower ACE inhibitory potency of food derived ACE inhibitory peptides.

Table 1: IC_{50} value of captopril and different *idli* batter samples

Sample ID	Sample description	IC_{50} value
1	Captopril	0.731 ng/ml
Batter 'S'	*Idli* batter (3:1 proportion fermented for 14 h at room temperature)	7.558 mg of protein/ml
Batter 'C'	*Idli* batter (3:1 proportion with 344 U of protease per gram of batter and fermented for 14 h at room temperature)	0.480 mg of protein/ml
Batter 'D'	*Idli* batter (2:1 proportion with 344 U of protease per gram of batter and fermented for 14 h at 37°C)	4.378 mg of protein/ml

Conclusion

Recently, consumer's preference towards naturally available bioactive compounds is found to be increasing because of no side-effects. Intake of bioactive compounds as one of the ingredient of daily dietary food is often effective in promoting health and reduction of risk of disease development. From the present work, it can be concluded that *idli* batter can serve as a potential source of ACE inhibitory activity. The conditions prevailing during *idli* batter fermentation such as temperature, proportion of black gram dhal used in preparation of batter, and addition of an extrinsic source of protease can all have an effect on ACE inhibition. Thus the ACE inhibitory activity of *idli* batter can be increased by addition of extrinsic protease to a greater extent than by increasing the dhal content in the batter. Though IC_{50} value of ACE inhibitory extract of *idli* batter was reduced on addition of extrinsic protease but still it was higher than that of captopril; hence *idli* batter derived peptides can be used as a preventive measure for hypertension rather than as a therapeutic drug.

Acknowledgments

The authors are thankful to Department of Biotechnology (DBT), India for funding this present work.

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