A Multistrategic Approach in the Development of Sourdough Bread Targeted Towards Blood Pressure Reduction

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Abstract

Rising prevalence of hypertension is pushing food industry towards the development of innovative food products with antihypertensive effects. The aim was to study the effect of reduced sodium content and 21% addition of wholemeal wheat sourdough (produced by *Lactobacillus brevis* CECT 8183 and protease) on proximate composition, GABA and peptide content of wheat bread. Angiotensin converting enzyme I (ACE) inhibitory and antioxidant activities were also evaluated. Sodium replacement by potassium salt did not affect chemical composition and biological activities of bread. In contrast, GABA and peptides <3 kDa contents in sourdough bread (SDB) were 7 and 3 times higher, respectively, than the observed in control. ACE inhibitory and antioxidant activities of the peptide fraction < 3 kDa from SDB was 1.7 and 2.6-3.0 times higher than control. Therefore, the combination of reduced sodium content with enriched concentrations of bioactive compounds in bread making may provide interesting perspectives for development of innovative breads towards blood pressure reduction.

Keywords: wheat bread, hypertension, sodium reduction, sourdough, antioxidant, angiotensin I converting enzyme

Abbreviations ACE angiotensin I converting enzyme; BP blood pressure; CB conventional wheat bread; GABA γ-aminobutyric acid; LAB lactic acid bacteria; LSB low-sodium wheat sourdough bread; MALDI-TOF matrix assisted laser desorption ionization time of flight; ORAC oxygen radical absorbance capacity; SDB low sodium wheat sourdough bread; SD sourdough; SDS-PAGE Sodium dodecyl polyacrylamide gel electrophoresis

Introduction

Hypertension is a public health problem associated with cardiovascular complications affecting 25% of adult population worldwide [1]. Reduction of sodium intake can shift the population distribution of hypertension [2]. Similarly, the consumption of healthy foods that contain bioactive compounds with antihypertensive activity may result effective in improving health outcomes of people with raised blood pressure (BP). Moreover, an increased intake of foods rich in antioxidants may ameliorate oxidative stress, and therefore, prevent from the development of hypertension and other cardiovascular complications [3]. Food industry must have an active role to solve this societal challenge through innovation focused on the development of foods with reduced sodium content and with antihypertensive and antioxidant properties. This approach will provide consumers with healthy foods that help in the prevention of hypertension.

Bread is considered to be the foodstuff that provides the most dietary salt (NaCl), therefore it is one of the key targets in any salt reduction strategy. A decrease in the salt content of bread is possible by replacing partially the salt content by other salts, mainly of potassium [4] without detrimental effects in terms of both technology and sensory acceptance [5]. Healthy and organoleptic features of bread can be additionally improved through sourdough fermentation by lactic acid bacteria (LAB) [6,7]. Recent studies have shown that LAB metabolism may produce new nutritionally bioactive compounds with antihypertensive activity such as γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the mammalian nervous system, that is well known for lowering BP and its diuretic and tranquilizer effects [8,9]. Angiotensin I converting enzyme (ACE)-inhibitory peptides and antioxidant peptides may be also synthesized by LAB during sourdough fermentation [10-12]. Inhibition of ACE hinders the formation of angiotensin II, a potent vasoconstrictor that results in BP decrease [13]. Antioxidant peptides may delay oxidative processes *in vivo* through radical scavenging, chelation of metal ion, inhibition of lipid peroxidation, etc [12]. To the best of our knowledge no literature data deal with the development of a functional wheat bread from an innovative multi-strategic approach consisting of partial replacement of salt by potassium citrate, protease addition and sourdough fermentation by *Lactobacillus brevis* CECT 8183 strain (isolated from Spanish cheese)

with proven high GABA-producing ability [14]. The effect of these approaches on nutritional composition, GABA and peptides production with ACE-inhibitory and antioxidant activities was evaluated.

Materials and Methods

Materials

Wholemeal wheat, white wheat and roasted soybean flour were purchased from Harinas Polo (Zaragoza, Spain). Protease enzyme was from Puratos S.A. (Groot-Bijgaarden, Belgium) and potassium citrate was purchased from Quimidroga S. A. (Barcelona, Spain). Sourdough and the three types of bread were made at pilot scale. Each loaf of bread was grated on a laboratory knife mill (Grandomix GM 200) at 7,000 rpm for 10 seconds and stored at -20 ºC.

Sourdough fermentation and bread preparation

Lactobacillus brevis CECT 8183 isolated from an artisan Spanish cheese and with high proven capacity for GABA synthesis [14] was used as a starter for the sourdough fermentation. Four cryobeads of the microorganism were cultivated on Man, Rogosa and Sharpe (MRS) broth medium (AES Chemunex, Terrassa, Spain). After 48 h at 30 ºC of incubation, the cells were recovered by centrifugation (10,000 x g for 15 min), washed twice in sterile water, and re-suspended in a 10 µM pyridoxal 5-phosphate solution at the minimum cell density of log 8 CFU/ml. Whole wheat flour (16.5%), soya flour (2%), protease (1.5%) and distilled water (80%) were used to prepare 5 L of sourdough (the dough yield was 500) with a continuous speed mixer (100 rpm) in a bioreactor (Biostat A plus, Sartorius, Germany) previously sterilized at 121 ºC for 20 min. Sourdough fermentation was carried out at 30 ºC for 48 h. In order to enhance GABA production, pH was maintained at 4.5 by the continuous addition of 2.5 M $KHCO_3$. At the end of fermentation, protease was inactivated by increasing the temperature to 70 ºC for 2 min. Sourdough fermentations were carried out ten times and analyzed in duplicate to check the GABA content.

Three types of bread (conventional wheat bread, CB; low-sodium wheat bread, LSB; low-sodium wheat sourdough bread, SDB) were manufactured according to the formulation shown in Table 1. After kneading and make-up, the bread dough was fermented at 27 ºC for 90 min with a humidity level of 76%. Baking was carried out at 205 ºC for 25 min.

Sodium docecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE)

Proteins in breads and sourdough were extracted following the procedure of Dupont et al. [15] and separated under non-reducing conditions by SDS-PAGE on NuPAGE Novex 4−12% Bis-Tris gels in the XCell-sure lock

84 minicell system (Invitrogen, Madrid, Spain) at 200V for 35 min. Proteins were stained with SimplyBlue SafeStain
85 (Invitrogen), followed by destaining in deionized water. The molecular weights of poly- and oligopeptides were
86 determined by comparison with the molecular weight marker® solution Sharp Novex unstained standard
87 (Invitrogen).
88 *Chemical Analysis*
89 Proximate composition of breads and sourdough was determined following AOAC [16] methods and they include:
90 moisture (method 925.10), protein (method 920.87), fat (method 922.06), ash (method 923.03) and total dietary
91 fiber (method 991.43). Carbohydrates were calculated by difference: 100 – (% water –% protein – % total fat – %
92 total ash – % total dietary fibre) [17]. Na and K were analysed by inductively coupled plasma atomic emission
93 spectroscopy (ICP-AES) using a Perkin-Elmer Optima 7300-DV spectrophotometer (Waltham MA, USA), following
94 microwave digestion of the samples with nitric acid and hydrogen peroxide. The extract obtained was then diluted
95 and absorbance was read at a wavelength of 589.59 nm for Na and 766.49 nm for K. Quantification of both
96 elements was performed by external calibration. GABA was determined by reverse-phase high performance liquid
97 chromatography, followed by UV detection after pre-column derivatization by 6-aminoquinolyl-N-hydrxy succinyl
98 carbamate (AccQTag) according to Cohen and Michaud [18]. For peptides determination, breads and sourdough
99 were ultrafiltrated through membranes of 3 kDa pore size (Millipore Corporation, Billerica, MA, USA). Permeates
100 were analyzed by the DC protein assay (Biorad) using BSA as standard.
101 *Biological Activity Testing*
102 Antioxidant activity was analyzed by the oxygen radical absorbance capacity (ORAC-FL) method [19] in
103 methanolic extracts and the water soluble 3 kDa peptide fraction of breads prepared according to Caceres et al.
104 [20] and Garcia-Mora et al. [19], respectively. ACE-inhibitory activity of the 3 kDa peptide fraction of water-soluble
105 extracts of samples was measured following the fluorescence-based protocol of Sentandreu and Toldrá [21]. IC_{50}
106 was determined by dose–response curves using the non-linear regression sigmoidal curve fit function in GraphPad
107 Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA).
108 *MALDI-TOF Analyses*
109 Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis was performed in a Voyager-DE
110 PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed 337 nm nitrogen laser (1
111 ns pulse width and 3 Hz frequency) which was operated in the reflectror mode for positive ions. The 3 kDa peptide

fraction of breads was mixed with the matrix at a ratio of 1:5 (v/v), and 1 µL of this solution was spotted onto a flat stainless-steel sample plate and dried in air. Mass spectra were obtained at m/z range 500-4000. External mass calibration was applied using the monoisotopic [M + H]+ values of des-Arg^1 Bradykinin, Angiotensin I, Insuline, Glu1-Fibrino-peptide B, adrenocorticotropic hormone fragments 1-17, 18-39, and 7-38. Parameters of the instrument were as follows: time of flight analyser (1.3 m flight path) with an acceleration voltage of 20 kV, 76% grid voltage, 0.001% ion guide wire voltage, and a delayed extraction time of 400 ns.

Statistical Analysis

Data were subjected to one-way analysis of variance (ANOVA) by Statgraphics Centurion XVI software, version 16.1.17 (Statistical Graphics Corporation, Rockville, Md). Differences between samples were compared by using a Duncan´s multiple-range test at $P \leq 0.05$ probability levels.

Results and Discussion

Proximate Composition, Protein and Peptide Profiles

The proximate composition (moisture, protein, fat, ash and carbohydrates) did not significantly differ ($P>0.05$) between breads (Table 2), with the exception of dietary fiber that was significantly lower in CB. Potassium content was 2-fold higher while sodium content was 1.5-fold lower in low sodium breads (LSB and SDB compared to control ($P\leq0.05$). These results are consistent with the replacement of sodium chloride by potassium citrate in the formulation of these breads.

GABA concentration in low-sodium wheat bread was similar ($P>0.05$) to control (Table 2), therefore, replacement of sodium by potassium salt did not influence GABA content in experimental breads. GABA is produced during dough mixing and yeast´s fermentation by conversion of glutamic acid by glutamate decarboxylase of wheat and yeast [22]. In our study, GABA concentration in SDB increased 7 times by addition of sourdough in bread formulation ($P\leq0.05$). GABA content in SD reached a concentration 15-fold higher than those previously reported for sourdoughs of wheat, pseudocereals and legume flours started with a pool of selected LAB [23,10]. Protease addition during sourdough fermentation increases the concentration of free amino acids [24] such as glutamine and glutamate improving the production yield of GABA during sourdough fermentation [25]. Additional protein degradation and glutamine and glutamate metabolism by lactobacilli glutaminases and glutamate decarboxilase give rise to GABA accumulation in sourdough [25]. GABA concentration in low-sodium SDB was much lower than the theoretical concentration expected from the addition of SD at a level of 21% (1230.8 mg/100g d.m.). GABA

losses have been reported during bread making due to its consumption by yeast during proofing and thermal degradation by Maillard browning reactions that take place during baking [22]. An additional degradation of GABA might have occurred during thermal inactivation of protease (70 ºC for 2 min) after sourdough fermentation. Human intervention studies have shown that a daily intake of 10-20 mg of GABA is able to prevent pre-hypertension [9,26]. The results of the present study suggest that a daily consumption of 100 g of bread (fresh weight) would provide enough GABA (22.4 mg) to display the health benefits observed in the previous studies.

Bioactive peptides with ACE inhibitory and antioxidant activity have a molecular weight below 3 kDa, therefore, the quantification of peptides < 3 kDa was carried out in this study. Results in Table 2 show that LSB and CB had a similar concentration of peptides < 3 kDa ($P>0.05$), unlike SDB with a concentration 3 times higher than those found in LSB and CB ($P<0.05$). Sourdough fermentation is known to cause an increase in the concentration of peptides compared with bread dough [27] due to proteolysis by LAB proteases and peptidases. The use of commercial proteases during wheat flour sourdough fermentation may result in a more extensive proteolysis and, subsequently, in an increase of peptides yield. SDS-PAGE shows that protease addition and SD fermentation by *L. brevis* CECT 8183 gave rise to intense protein bands with a range of molecular masses between 30-40 and 58-70 kDa and small protein fragments at molecular masses below 20 kDa (Fig. 1; lane 4). In spite of no differences were observed by SDS-PAGE in the protein profile of SDB compared to LSB and CB (Fig. 1, lanes 1-3), a different peptide profile was found by MALDI-TOF between 3 kDa permeates from SDB and its respective control (LSB) (Table 3). Bolded peptide masses at m/z 519, 523, 537, 544, 549, 565, 609, 610, 613, 713, and 723 were only found in SDB, therefore, they could be released during sourdough fermentation. Comparing our results with reported SDS-PAGE profiles [28] and peptide content [10,28] of wholemeal wheat sourdoughs fermented by different LAB strains (13.0-22.5 mg/g of sourdough d.m., Table 2) we found that addition of commercial protease during sourdough fermentation resulted in a more extensive proteolysis and a markedly enrichment of peptides < 3 kDa (57.7 mg/g of sourdough d.m.). Peptide concentrations in SDB (11.3 mg/g d.m., Table 2) were slightly lower than the theoretical concentration expected from the addition of SD at a level of 21% (12.7 mg/g d.m.). This lower peptide concentration could be due to their degradation as consequence of Maillard reactions that take place during baking [29] and at the end of sourdough fermentation. Zhao et al. [29] reported a decrease in peptide content due to LAB enzymatic activity at the proofing and kneading stages. In our study this effect was unlikely to occur since a thermal treatment at 70ºC for 2 min was applied for enzymatic inactivation.

ACE Inhibitory and Antioxidant Activities

Table 4 shows ACE inhibitory and antioxidant activities of 3 kDa permeates of water-soluble extracts obtained from sourdough and experimental breads. ACE inhibitory and antioxidant activity of the peptide fraction < 3 kDa from SDB was 2 times and up to 3 times higher, respectively than those of LSB and CB ($P \leq 0.05$). These results are due to bread supplementation with SD in which the release of amino acid sequences with antioxidant and ACE inhibitory activities from grain storage proteins may occur by protease and peptidase activities [27,11,12]. Positive correlation between peptide content and ACEI inhibitory or antioxidant activities of breads supports this consideration (Table 4).

ACE inhibitory activity of the peptide fraction < 3 kDa from SD (0.09 mg peptide/mL) was between 2.1-26 times higher than those reported previously (0.19-0.54 mg/mL) for peptide fractions from wheat sourdoughs started by a pool of selected LAB [10]. Type of wheat grain variety, composition of starter culture, addition of protease, dough yield and processing conditions (fermentation time and temperature) could explain these differences. The comparison of IC_{50} values of 3 kDa permeates from SD (94.8 µg/mL) and SDB (127.2 µg/mL) indicated that ACE inhibitory activity remained stable after kneading, proofing and baking stages. Therefore, it can be assumed that ACE inhibitory peptides released during sourdough fermentation were not further degraded as consequence of yeast metabolism and thermal treatment in agreement with previous findings [29]. No previous studies have addressed the impact of bread making on the antioxidant activity of peptides.

Clinical trials with hypertensive humans indicate that consumption of 3-6 mg of peptides derived from milk proteins with ACE-inhibitory activity per day reduces systolic BP [13]. Although SDB was not evaluated *in vivo*, an initial assessment of their bioactivity can be made based on the comparison of IC_{50} (127.23 µg/mL) with the theoretical concentration of peptides reached in blood after consumption of one serving of fresh SDB. One serving of SDB (100 g fresh weight) contains 723 mg of peptides <3 kDa, therefore, it can be assumed that this amount may reach the absorption site in the intestine and further the blood stream at a concentration 2 times higher than the IC_{50} value of 3 kDa peptide fraction (241 µg/mL) in a reference person containing approximately 3 L of plasma. Although gastrointestinal digestion, absorption, transport and metabolism are likely to impact ACE inhibitory activity of bioactive peptides, results from the present study are promising and deserve further research to evaluate the antihypertensive effect of SDB *in vivo*.

Antioxidant activity was also analyzed in acidified methanolic extracts from sourdough and breads (Table 4). It is worth noting that ORAC values of methanolic extracts were higher than those found for the ≤3 kDa peptide fraction. Higher antioxidant capacity of methanolic extracts could be attributed to a better solubility and extraction of compounds with antioxidant activity such as phenolics [30]. Similarly to results found in the peptide fraction < 3 kDa, bread supplemented with 21% wheat sourdough gave rise to ORAC values 1.5 and 1.9-fold higher than the observed in LSB and CB, respectively (Table 4). Cereal fermentation by LAB result in an increase and bioconversion of free phenolic compounds [31], effect that could be related with the increased ORAC values observed in SDB. Cereal and LAB enzymes are key contributors on the conversion of phenolic acids during fermentation [32] which have been found directly related to an increased antioxidant activity in fermented products [33]. Comparing SD to SDB, it is worth noting that the bread making process seems to have no impact on the antioxidant activity. However, the synthesis of substances with antioxidant properties, including certain Maillard reaction products that are accumulated in the bread crust, may mask the real decrease of antioxidants in bread as well as any loss in total antioxidant activity [34].

Conclusions

This study shows that bread supplemented with wheat sourdough produced by *L. brevis* CECT 8183 and a commercial protease markedly improved the total antioxidant activity of bread and its content in GABA and small peptides (<3 kDa) with ACE-inhibitory and antioxidant activity. Therefore, the combination of reduced sodium content with enriched concentrations of bioactive compounds such as GABA, ACE inhibitory and antioxidant peptides may provide interesting perspectives for development of innovative breads aimed at reducing blood pressure.

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Table 1 Formulas used for manufacture of conventional wheat bread (CB), low-sodium wheat bread (LSB), low-sodium sourdough bread (SDB).

Ingredients (%)	CB	LSB	SDB
Wheat flour	62.62	62.62	60.09
Water	35.07	35.07	16.82
Sodium chloride	1.13	0.81	0.78
Baker´s yeast	0.94	0.94	0.90
Improver	0.25	0.25	0.24
Sourdough	-	-	21.03
Potassium citrate	-	0.31	0.13

Table 2 Proximate composition and GABA content of conventional wheat bread (CB), low-sodium wheat bread (LSB), low-sodium wheat sourdough bread (SDB) and sourdough (SD).

	CB	LSB	SDB	SD
Humidity (%)	35.93 ± 0.31^{a}	35.53 ± 0.35^{a}	36.00 ± 0.44^{a}	84.93 ± 0.15^{b}
Protein (%, d.m.)	13.89 ± 0.14^{b}	13.78 ± 0.14^{b}	14.06 ± 0.16^{b}	18.10 ± 0.45^{a}
Fat (%, d.m.)	2.00 ± 0.14^{b}	1.93 ± 0.12^{b}	1.95 ± 0.15^{b}	5.76 ± 0.87^{a}
Ash (%, d.m.)	2.66 ± 0.06^{b}	2.48 ± 0.12^{b}	2.49 ± 0.13^{b}	6.62 ± 0.81^{a}
Total Fiber (%, d.m.)	3.15 ± 0.19^{b}	4.03 ± 0.25^{c}	3.97 ± 0.23^{c}	< 3.31^{a}
Carbohydrates (%, d.m.)	78.30 ± 0.47^{b}	77.78 ± 0.67^{b}	77.53 ± 0.44^{b}	69.29 ± 1.71^{a}
Potassium (mg/100 g d.m.)	205.50 ± 14.50^{a}	399.15 ± 18.18^{b}	420.31 ± 23.02^{b}	3944.81 ± 53.12^{c}
Sodium (mg/100 g d.m.)	817.86 ± 12.39^{c}	576.50 ± 13.19^{b}	557.81 ± 18.94^{b}	< 66.22^{a}
GABA (mg/100 g d.m.)	4.99 ± 0.07^{a}	5.18 ± 0.10^{a}	35.05 ± 1.30^{b}	5860.93 ± 176.59^{c}
Peptides <3kDa (mg/g d.m.)	3.64±0.12^{a}	3.62±0.26^{a}	11.30±0.85^{b}	57.70±3.62^{c}

Data indicate mean value ± standard deviation of three independent experiments. Means with different letters within a row are significantly different ($P \leq 0.05$, Duncan test).

Table 3 Peptide masses[1] profile by MALDI-TOF found in 3 kDa permeates from low-sodium wheat bread (LSB) and low-sodium wheat sourdough bread (SDB)

Ion mass (m/z)	Relative Intensity (%)	
	LSB	SDB
509	22.82	52.25
519	nd[2]	23.63
523	nd	23.93
527	52.37	61.17
528	42.37	44.13
529	31.5	34.27
530	41.61	34.93
537	nd	42.56
542	26.21	32,32
544	nd	31.6
548	25.4	34.89
549	nd	29.23
550	32.36	31.17
553	19.39	37.61
555	29.73	46.09
556	33.5	37.54
565	nd	34.46
568	60.38	50.03
569	30.91	33.12
609	nd	28.15
610	nd	30.5
613	nd	23.96
650	48.46	41.96
656	100	64.01
657	39.99	25.54
659	88.85	nd
660	45.87	nd
666	45.25	35.77
672	95.68	100
673	32.9	45.3
687	30.23	43.85
713	nd	24.63
723	nd	22.51
861	44.62	32.48
877	51.37	38.57

[1]Peptides masses with intensities ≥ 20%; [2] nd: not detected. Bolded ion masses were only found in SDB

Table 4 Peptide concentration, ACE-inhibitory (calculated as % inhibition and IC_{50} value) and antioxidant activities (measured by ORAC) of 3 kDa permeate from conventional wheat bread (CB), low-sodium wheat bread (LSB) and low-sodium wheat sourdough bread (SDB) and sourdough (SD).

Sample	Peptide fraction < 3 kDa			Methanolic extract
	ACE inhibition (%)	IC_{50} (µg peptide/mL)	ORAC (µM TE)	ORAC (µmol TE/g d.m.)
CB	10.29±0.81[a]	258.27±30.46 [b]	474.98±29.23[a]	2090.37 ± 238.06[a]
LSB	10.29±0.81[a]	212.47±17.51[b]	555.14±58.53[a]	2646.27 ± 350.77[a]
SDB	68.18±0.79[b]	127.23±15.77[a]	1461.61±77.19[b]	4018.96 ± 450.88[b]
SD	95.13±0.15[c]	94.80±10.80[a]	8317.81±731.46[c]	18217.13 ± 890.94[c]
Pearson r[#]	0.8327*		0.9984*	
P-value	0.0003		0.0000	

Data indicate mean value ± standard deviation of three independent experiments. Means with different letters within rows are significantly different (P ≤ 0.05, Duncan test).[#]Pearson r correlation coefficient between peptide concentration and ACE inhibition or ORAC in the 3 kDa permeate. * Significant correlation, P<0.05.

Fig. 1 SDS-PAGE profile of protein extracts prepared from conventional bread (CB, lane 2), low-sodium wheat bread (LSB, lane 3), low sodium wheat sourdough bread (SDB, lane 4) and sourdough (SD, lane 5). Molecular weight standards are shown in lane 1.