Food Chemistry 142 (2014) 166–169

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

Iron-binding properties of sugar cane yeast peptides

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ARTICLE INFO

Article history:
Received 7 February 2013
Received in revised form 25 June 2013
Accepted 28 June 2013
Available online 18 July 2013

Keywords:
Sugar-cane yeast
Iron-chelating peptides
Dialyzability
Immobilized metal affinity chromatography
Protein hydrolysates

ABSTRACT

The extract of sugar-cane yeast (*Saccharomyces cerevisiae*) was enzymatically hydrolysed by Alcalase, Protex or Viscozyme. Hydrolysates were fractionated using a membrane ultrafiltration system and peptides smaller than 5 kDa were evaluated for iron chelating ability through measurements of iron solubility, binding capacity and dialyzability. Iron-chelating peptides were isolated using immobilized metal affinity chromatography (IMAC). They showed higher content of His, Lys, and Arg than the original hydrolysates. In spite of poor iron solubility, hydrolysates of Viscozyme provided higher iron dialyzability than those of other enzymes. This means that more chelates of iron or complexes were formed and these kept the iron stable during simulated gastro-intestinal digestion *in vitro*, improving its dialyzability.

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1. Introduction

The characteristics and properties of peptides released by the controlled enzymatic treatment of proteins vary mainly according to the specificity of the enzyme. Various proteins have been used as a source for producing biologically active peptides. Milk and meat proteins have been reported as precursors of peptides with mineral binding abilities (Storcksdieck, Bonsmann, & Hurrell, 2007; Vegarud, Langsrud, & Svenning, 2000).

Iron deficiency is the most common nutritional disorder in the world, generally resulting from insufficient intake, altered metabolism or impaired absorption caused by the interference of other dietary factors (WHO/FAO, 2004). Food fortification is the most practical and best long-term strategy to prevent iron deficiency in the population.

The iron from $FeSO_4$ salt, commonly used as a supplement or for food fortification, is absorbed like the nonheme iron present in food components of the diet (Layrisse, Martínez-Torres, Cook, Walker, & Finch, 1973). However, poor taste and low bioavailability are issues that need to be resolved. Iron chelate compounds represent an alternative, since they change the chemical and physical characteristics of the iron, providing more stability within the metallic molecule formed (Ashmead, 2001). Proteolytic *in vitro* digestion can release peptides that are able to bind iron, an ability associated with certain specific amino acids such as histidine, lysine, cystine, aspartic or glutamic residues (Ou et al., 2010; Wu, Liu, Zhao, & Mingyong, 2012). In general, they are amino acids with functional groups capable of forming coordinated covalent bonds.

Yeasts are known to be an excellent source of proteins, B vitamins, essential minerals and dietary fibers for human consumption worldwide, as single-cell protein or as components of traditional foods. Amongst yeast species, *Saccharomyces cerevisiae* is fully accepted in foods (Bekatorou, Psarianos, & Koutinas, 2006). The cell protein content ($N \times 5.78$) can reach approximately 50% (dry basis) and the essential amino acids profile with respect to lysine, tryptophan and threonine is nutritionally satisfactory for humans (FAO/WHO/UNU, 2007). Yeast extract is produced by enzymatic digestion of the cell wall and it results in a soluble material containing peptides, free amino acids, nucleotides, vitamins, minerals and oligosaccharides (Pacheco, Caballero-Córdoba, & Sgarbieri, 1997).

The motivation for using yeast extract as a source of peptides for chelating iron is based on two main considerations: the enormous potential availability of the raw material and its high biological quality protein. From the nutritional point of view, our main goal is to obtain natural products that can increase iron absorption and bioavailability to meet the needs of patients with iron deficiency anemia, mainly children and pregnant women. Specifically, the objectives of this study were to obtain a fraction of small-size

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0308-8146/$ - see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.foodchem.2013.06.133

peptides from enzymatic yeast hydrolysates and to investigate their ability to bind iron and their influence on iron bioavailability.

2. Material and methods

2.1. Material

Sugar-cane yeast extract (*S. cerevisiae*) was provided by a Brazilian sugar cane processing plant. Pepsin, pancreatin, and bile extracts were purchased from Sigma–Aldrich (St. Louis, MO, USA). Stock standard solution of iron (1000 mg/mL) was from Merck (Merck KGaA, Germany). Chemical and solvents used were of analytical and HPLC grade.

2.2. Preparation of enzymatic hydrolysates, degree of hydrolysis (DH) and fractionation

The yeast extract was hydrolysed by Alcalase (from *Bacillus licheniformis*, activity 2.4 AU/g), Viscozyme L (from *Aspergillus aculeatus,* 100 FBG/g) from Novozymes (Novozymes Latin America Limited), and Protex 51FP (*Aspergillus oryzae,* 400,000 HU/g) from Genencor (Division of Danisco, Japan), using a Metrohm 716 pH-stat (Les Ulis, France) at 10% (w/v) substrate concentration. DH was calculated as recommended by Adler-Nissen (1979). The best conditions for hydrolysis were obtained from an experimental Rotatable Central Composite Design (RCCD 2^2), where a set of 11 trials including three central points was employed. The independent variables were pH and the enzyme/substrate (*E*/*S*) ratio. The measured variable response was the degree of hydrolysis.

Fractionation was accomplished using an ultrafiltration system and Prep/Scale™ TFF Cartridges with nominal cut-off of 5 kDa (Pellicon® Millipore Bedford, MA, USA). Fractions containing molecules smaller than 5 kDa were freeze-dried and stored at −20 °C until used.

2.3. Determination of amino acids

Amino acids were determined by reverse phase-high performance liquid chromatography (RP-HPLC) using a Shimadzu HPLC system (Shimadzu Corporation, Japan), equipped with a Luna/Phenomenex C18 column (4.6× 250 mm, 5 μ). Identification and quantification was done by external standard (Pierce/PN 20088) with UV detection at 254 nm (Hagen, Frost, & Augustin, 1989; White, Hart, and Fry,1986).

2.4. Iron solubility

The method of Kim et al. (2007) was used to measure iron solubility. The freeze-dried samples of yeast extract hydrolysates were dissolved in milli-Q water for testing. Mineral iron was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Vista MPX, Varian, Mulgrave, Australia). Iron solubility was expressed as a percentage of the total iron-ion contents (initially added), and was calculated as Iron solubility% = [(iron−ion supernatant)/(iron–ion total]× 100.

2.5. Iron binding capacity

The iron binding capacity of the hydrolysates was measured according to the method of Wang et al. (2011) with slight modifications. First, an iron binding reaction was performed to produce complexes. Hydrolysates (fractions <5 kDa) with 1% (w/v) in protein content, pH adjusted to 5.5, were mixed with iron as $FeSO_{4(s)}$ 0.1% (w/v), both in aqueous solution. Incubation was performed in a shaking water bath for 30 min at 40 °C. Then, the solution was diluted 1:50 (v/v) in milli-Q water and dialyzed during 48 h at 26 °C, against milli-Q water, for the removal of free iron ions, using a Spectra/Por® dialysis membrane with a cut-off of 500 Da, (Spectrum Laboratories, Inc., CA, USA). A blank without hydrolysates was run in parallel to samples and dialyzed. After dialysis, the retentate containing iron bound to peptides (complexes) was analysed for iron content by ICP. The percentage of iron binding capacity was calculated as:

Iron binding capacity % = [iron content in complex (g)/total iron in solution before binding (g)]× 100.

2.6. Iron bioavailability estimated through in vitro dialyzability

In vitro dialyzability was used to predict iron bioavailability of hydrolysates (fraction <5 kDa). Dialyzability involves a two-stage (gastric and intestinal) simulated digestion and a dialysis. The procedure is similar to that described by Argyri, Birba, Miller, Komaitis, and Kapsokefalou (2009), a setup which allows the rapid and efficient application of the dialyzability method. The simulated gastrointestinal digestion occurs in six-well plates with inserts and Spectra/Por® dialysis membranes (cut-off of 6000–8000 Da) tightly held in place with elastic bands. The percentage of iron dialyzability was calculated as: [(dialyzable iron)/(total iron)] × 100. Dialyzable iron was the iron that passed through the dialysis membrane during the *in vitro* digestion. Dialyzable iron was the iron content of the dialysate, and the total iron, the amount of iron added to the sample material prior to digesting (final concentration of 0.2 mM).

2.7. Separation of iron chelating peptides by IMAC-Fe (III)

A chromatographic column with IMAC Sepharose High Performance (IMAC-HP) resin (GE Healthcare Bio-Science AB, Sweden) installed in a low pressure liquid chromatography system (FPLC) from Pharmacia (Amersham Pharmacia Biotech) was used to separate the iron chelating peptides. The method of Lv et al. (2009) was followed with some modifications. A column was packed with IMAC-HP (10 mL) and charged with Fe^{3+} (5 mL of 200 mM $FeCl_3$). After washing the unbound iron out of the column with milli-Q water (5 bed volumes), the nonspecific bound iron was removed with 50 mM sodium acetate-acetic acid buffer (NaAc/HAc), pH 3.6 (2–5 vol), and the column was equilibrated using the same buffer. Subsequently, 3 mL of yeast extract hydrolysate solution <5 kDa (20 mg/mL in protein content) was loaded onto the column. Peptides without affinity to immobilized iron in the column were eluted with the equilibration buffer (50 mM NaAc/HAc). Then, the bound peptides were eluted using 100 mM $NH_4H_2PO_4$ solution, pH 4.5, and collected for further lyophilization. The absorbance of eluates was monitored at 280 nm. The flow rate was 1 mL/min for loading samples, and 2 mL/min for elution. Regeneration of the column was achieved with 50 mM EDTA, overnight. All chromatographic experiments were carried out at room temperature (24 °C). Iron-chelating peptide fractions isolated by IMAC were concentrated under nitrogen gas and lyophilized. Finally, they were characterized by amino acid profiling.

2.8. Statistical analysis

Analyses were performed in triplicate. Data were expressed as means ± standards deviations (SD) and compared using analysis of variance (ANOVA) and the Tukey test. Statistical analysis was performed using the STATISTICA 7 software package for Windows (StatSoft, Inc., Tulsa, OK, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Physicochemical characterisation of the yeast extract

The yeast extract utilised in this study consisted of 3.35% moisture, 13.88% ash, 62.82% proteins (conversion factor 5.78), 0.15% lipids and 19.80% carbohydrates.

3.2. Enzymatic hydrolysates, degree of hydrolysis (DH)

The best conditions for hydrolysing the yeast extract were defined by performing a 2^2 RCCD experiment whose results are shown in Table 1.

3.3. Iron-chelating peptides isolated by IMAC-Fe(III)

The peptide elution profile obtained by using IMAC-Fe(III) technique confirms that there is a fraction of peptides from yeast extract hydrolysates with high ability to chelate iron. The elution profile of Viscozyme hydrolysate is shown in Fig. 1. Peptides without affinity eluted with the equilibration buffer (F1). The peptides with affinity were retained in the column matrix, bound to the immobilized iron, and were eluted with the 100 mM $NH_4H_4PO_4$ buffer. Similar profiles were obtained for the other two enzymes (Alcalase and Protex).

3.3.1. Amino acid profile

The amino acid profile for each of the yeast extract hydrolysates smaller than 5 kDa and the respective iron-chelating fractions isolated by IMAC were obtained by RP-HPLC (data not shown). Matching the amino acidic composition of peptides in hydrolysates and chelated peptides for the same enzymatic treatment, we observed that chelated peptides from Alcalase hydrolysates were rich in His, Lys and Arg residues, whilst those from Protex and Viscozyme hydrolysates were rich in His, Lys, Arg, and Gly residues. Table 2 shows the enrichment of amino acids in the chelated fractions related to original hydrolysates, total fraction.

The iron chelating capacity of residues His, Lys and Arg in peptides has been reported by other researchers (Choi, Kim, Lim, & Lim, 1998; Kim et al. 2007; Swain, Tabatabai, & Reddy, 2002). In the present work it is not so well understood why the presence of these residues favours the chelation of iron in IMAC-Fe(III) preferentially to Asp or Glu.

3.4. Iron solubility and iron binding capacity

Iron solubility is considered one of the main requirements for promoting a high availability of iron. The solubility of iron plus yeast extract was 20.2 ± 0.8%. This value was lower than that obtained for enzymatic hydrolysates (Table 3), indicating that peptides have a greater number of binding sites for iron than the original yeast extract material. Iron solubility in yeast extract hydrolysates (fractions <5 kDa) ranged from 34% to 40% (Table 3). This low iron solubility may be ascribed to interference of carbohydrates compounds present in the samples, rendering the iron insoluble. Apparently this effect was more evident when theViscozyme was utilised. Viscozyme, a multi-enzyme complex, differs from the other two enzymes in that it contains a wide range of carbohydrases including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase. It is probable that this multi-enzyme complex acting on the indigenous carbohydrates present in the yeast hydrolysates allowed them to sequester the iron, causing decreasing in iron solubility.

The iron-binding capacity as defined in the method of Wang et al. (2011) represents the iron bound to peptides forming complexes or chelates once free iron is eliminated by dialysis. After 48 h of dialysis, the iron binding capacity of the blank-corrected Alcalase hydrolysate was found to be significantly higher than that of the Viscozyme and Protex hydrolysates, but no correlation was observed with iron solubility (Table 3). When the hydrolysates were incubated with iron in a Wang system they acquired a cloudy appearance indicating the loss of solubility. This turbidity however was eliminated by diluting the sample 50-fold and the dialysis allowed to proceed.

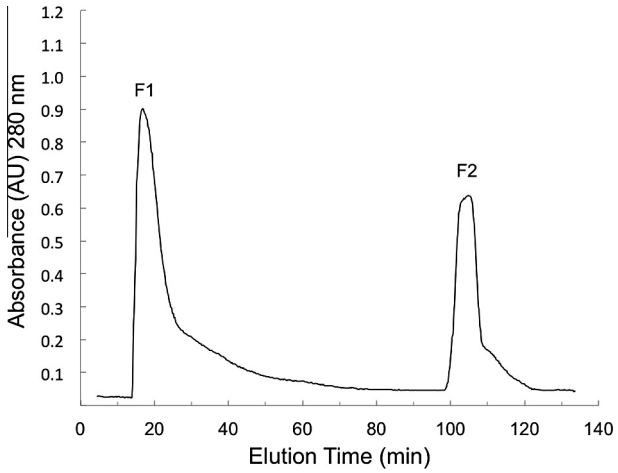

Fig. 1. IMAC-Fe(III) elution profile of peptides (fraction <5 kDa) from yeast extract Viscozyme hydrolysate. The peptides with little or no ability to bind iron eluted with the equilibration buffer (50 mM NaAc/HAc, pH 3.6) in peak F1. The peptides bound to iron immobilized in the IMAC column were eluted with the 100 mM $NH_4H_2PO_4$ elution buffer in peak F2. Viscozyme chelating sepharose high performance (IMAC-HP) was used with ferric as ligand.

Table 2
Alteration in the amino acid content of peptides with iron affinity in relation to the <5 kDa fraction. Lower values refer to the total fraction, and higher values to the retained peak.

Enzyme	Variation of amino acid content (g/100 g protein)			
	GLY	HIS	LYS	ARG
Alcalase	–	2.2–9.3	7.8–29.6	5.5–15.9
Protex 51FP	5.2–8.5	2.2–9.8	8.0–24.0	5.6–14.5
Viscozyme	5.2–7.3	2.1–7.5	7.5–15.6	5.4–13.9

Abbreviations: GLY, glycine; HIS, histidine; LYS, lysine; ARG, arginine.

Table 1
Conditions for hydrolysing yeast extract using pH-Stat system, and degree of hydrolysis (DH).

Enzyme	pH	Ratio E/S[a] (%)	Temperature (°C)	DH (%)
Alcalase	8.0	1.4	55	16.6
Protex 51FP	7.6	1.6	50	9.7
Viscozyme	4.4	2.0	51	15.8

[a] *E/S*, ratio enzyme/substrate.

Table 3
Percentage of iron solubility and iron-binding capacity of yeast extract hydrolysates (Means ± SD, $n = 3$, $P < 0.05$).

Yeast extract hydrolysates	% Iron solubility ± SD	*% Iron-binding capacity ± SD
Alcalase	40.4 ± 2.0[a]	26.3 ± 0.7[a]
Protex 51FP	40.9 ± 4.7[a]	21.4 ± 1.5[b]
Viscozyme	34.3 ± 3.7[b]	22.8 ± 0.1[b]

[a,b] Different letters (column) indicate statistically significant differences.
* % Iron-binding capacity blank corrected.

Table 4
Iron dialyzability of yeast extract hydrolysates <5 kDa (Means ± SD, $n = 3$, $P < 0.05$).

Yeast extract hydrolysate	% Dialyzability Fe ± SD
Alcalase	21.0 ± 0.1[c]
Protex 51FP	23.2 ± 2.1[c]
Viscozyme	35.3 ± 1.4[a]
0.075 M PIPES (control)	26.4 ± 0.5[b]

[a,b,c] Different letters indicate statistically significant differences.

The lack of correlation between peptide-bound iron solubility and iron-binding capacity can be seen when the lowest solubility of the Viscozyme hydrolysate is in accordance with its low binding capacity, but the high solubility of the Protex hydrolysate fails to match its low binding capacity. Therefore, the lack of a systematic interpretation of these results should be attributed to the inherent differences in the nature of the different enzymes.

3.5. Iron bioavailability

The iron bioavailability of the yeast extract hydrolysates was estimated by the iron dialyzability during *in vitro* digestion. The results are shown in Table 4. Of the three hydrolysates tested, only Viscozyme hydrolysate showed a percentage of iron dialyzability higher than that of the control.

Higher dializability normally would indicate that higher amounts of soluble and stable iron remain as such until the time of intestinal digestion. The different dialyzability values observed amongst hydrolysates is indicative therefore of the specificity of each enzyme to produce peptides with different iron-binding abilities.

4. Conclusions

Due to its better iron-binding properties of its hydrolysates, the Viscozyme appeared to be the enzyme of choice, as compared to Alcalase and Protex. The role of the constituting Viscozyme will remain obscure until further studies can show if this multi-enzyme complex has any relevance on the different results observed.

Acknowledgements

The authors acknowledge financial support from Fundação de Amparo a Pesquisa de São Paulo (FAPESP).

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