Short communication

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PII: S0308-8146(12)00597-3
DOI: 10.1016/j.foodchem.2012.03.112
Reference: FOCH 12405

To appear in: *Food Chemistry*

Received Date: 25 November 2011
Revised Date: 13 March 2012
Accepted Date: 26 March 2012

Please cite this article as: Torres-Fuentes, C., Alaiz, M., Vioque, J., iron-chelating activity of chickpea protein hydrolysate peptides, *Food Chemistry* (2012), doi: 10.1016/j.foodchem.2012.03.112

IRON-CHELATING ACTIVITY OF CHICKPEA PROTEIN HYDROLYSATE PEPTIDES

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ABSTRACT

Chickpea-chelating peptides were purified and analyzed for their iron-chelating activity. These peptides were purified after affinity and gel filtration chromatography from a chickpea protein hydrolysate produced with pepsin and pancreatin. Iron-chelating activity was higher in purified peptide fractions than in the original hydrolysate. Histidine contents were positively correlated with the iron-chelating activity. Hence fractions with histidine contents above 20% showed the highest chelating activity. These results show that iron-chelating peptides are generated after chickpea protein hydrolysis with pepsin plus pancreatin. These peptides, through metal chelation, may increase iron solubility and bioavailability and improve iron absorption.

Keywords: Chelating peptides; Chickpea; Iron; Protein hydrolysates; Pepsin; Pancreatin.

1. Introduction

Iron is an essential element in human nutrition. It participates in many biochemical processes, including electron transfer reactions, gene regulation, binding and transport of oxygen, and cell growth and differentiation. Iron deficiency may lead to diseases, such as anemia, glossitis, angular stomatitis, koilonchia, blue sclera, and esophageal webbing. Other physiological manifestations of iron deficiency include pregnancy complications, pica, increased absorption of lead and cadmium, alteration in drug metabolism, increased insulin sensitivity and impaired immune and mental function, physical performance and thermoregulation (Beard, 2001). Also, iron may generate reactive oxygen species (ROS) and be implicated in cardiovascular and neurological diseases, such as atherosclerosis, and Alzheimer's and Parkinson's diseases (Blat, Weiner, Youdim, & Fridkin, 2008). Also, ROS may have a negative impact on flavour, texture, nutritive value and shelf life of food products (Chung, Chang, Chao, Ching-Fwu, & Su-Tze, 2002). Thus, chelating agents that decrease free iron and favour iron bioavailability may possess therapeutic potential and prevent its pro-oxidant effects.

Some dietary compounds, such as reducing components, stearic acid, certain amino acids (His, Glu, Asp, and Cys), peptides released during proteolytic digestion and the so called "meat factor", enhance iron absorption (Swain, Tabatabai, & Reddy, 2002). These compounds may bind iron, forming soluble complexes and improving iron bioavailability (Glahn, & Van Campen, 1997; Storcksdieck, Bonsmann, & Hurrel, 2007). Also, amino acids and certain

other organic acids, increase iron absorption by buffering the pH of the intestinal contents (Van Campen, & Gross, 1969).

Different studies have shown the beneficial effect on iron absorption of peptides produced by enzymatic hydrolysis of vegetable proteins, such as soybean (Murray-Kolb, Welch, Theil, & Beard, 2003; Macfarlane et al, 1990; Baynes et al., 1990; Fidler, Davidsson, Walczyk, & Hurrell, 2003). Also, beef peptide fractions, purified by copper affinity chromatography, increase iron solubility, indicating that copper-chelating peptides also possess affinity for iron (Swain et al., 2002). Also, copper has been used as a ligand to purify lactoferrin, an iron-binding protein (Lönnerdal, Carlsson, & Porath, 1977).

In a previous work, we have purified, by metal affinity chromatography, copper-chelating peptides from a chickpea protein hydrolysate produced with pepsin plus pancreatin (Torres-Fuentes, Alaiz, & Vioque, 2011). The objective of the present work was to determine if chickpea copper-chelating peptides also possessed iron-chelating activity that may be of interest from a nutritional point of view.

2. Materials and methods

2.1. Chemicals

Diethyl ethoxymethylenemalonate was purchased from Fluka (Buchs, Switzerland). Amino acid standards, D,L-α-aminobutyric acid, trinitrobenzenesulphonic acid (TNBS), ethylendiamine tetra-acetic acid (EDTA), enzyme complexes of pepsin, pancreatin and [4,4'-[3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl] bisbenzenesulfonic acid] (ferroxine) were provided by Sigma-Aldrich.

2.2. Purification of chickpea-chelating peptides

Chelating peptides were purified as previously described (Torres-Fuentes et al., 2011). Chickpea protein isolates were hydrolyzed with the digestives enzymes pepsin and pancreatin. Then, chelating peptide fractions were purified from this hydrolysate by metal affinity chromatography, using immobilized copper, and chelating peptides were further fractionated by gel filtration chromatography (Figure 1).

2.3. Amino acid analysis

Amino acids were determined after derivatization with diethyl ethoxymethylenemalonate by HPLC, using D,L-α-aminobutyric acid as an internal standard (Alaiz, Navarro, Girón, & Vioque, 1992). The HPLC system consisted of a model 600E multisystem with a 484 UV-vis detector (Waters Corporation, Milford, MA, EEUU) equipped with a 300 mm _ 3.9 mm i.d. reversed-phase column (Novapack C_{18}, 4 m; Waters). A binary gradient was used for elution with a flow of 0.9 ml/min. The solvents used were (A) sodium acetate (25 mM), containing sodium azide (0.02% w/v), pH 6.0, and (B) acetonitrile. Elution was as follows: time, 0.0-3.0 min; linear gradient from A/B (91/9) to A/B (86/14); 3.0-13.0 min, elution with A/B (86/14); 13.0- 30.0 min, linear gradient from A/B (86:14) to A/B (69/31); and 30.0- 35.0 min, elution with A/B (69/31). The column was maintained at 18 °C. Derivatized amino acids were detected at 280 nm.

2.4. Determination of iron-chelating activity

Fe^{2+}-chelating activity was determined by measuring the formation of the Fe^{2+}-ferrozine complex (Carter, 1971). Samples (100 µg) were mixed with 250 µl of

100 mM Na acetate buffer, pH 4.9, and 30 µl of $FeCl_2$ (0.01%, w/v). Ferrozine (12.5 µl, 40 mM) was added after incubation for 30 min at room temperature. EDTA was used as a positive control. Binding of Fe(II) ions to ferrozine generates a coloured complex that was measured at 562 nm, using a microplate reader (Multiskan Spectrum, ThermoLab Systems, MA, USA).

Iron-chelating activity was calculated as:

% Chelating Activity = [(Abs control – Abs sample / Abs control)] x 100

3. Results and discussion

In a previous work (Torres-Fuentes *et al*., 2011), we have described the purification of chickpea copper-chelating peptides. The purification process is shown in Figure 1. A chickpea protein isolate was hydrolyzed sequentially with the digestive enzymes, pepsin and pancreatin. The resulting protein hydrolysate was used for the purification of chelating peptides. These peptides were purified by affinity chromatography, with immobilized copper, plus gel filtration chromatography. Fifteen chickpea-chelating peptide fractions were obtained.

Iron is a transition metal with characteristics similar to those of copper, and therefore, previously purified copper-chelating peptides may, as well, possess iron-chelating activity. To test this hypothesis, iron-chelating activity of purified peptide fractions was studied. Figure 2 shows the iron-chelating activity of the original chickpea protein hydrolysate, chickpea peptide fractions (F1, F2 and F3), purified after copper affinity chromatography, and EDTA. F1, F2 and F3 fractions showed higher iron-chelating activity than did the protein hydrolysate. Contrary to the observations with copper (Torres-Fuentes *et al*., 2011), no

139 correlation between the histidine contents and the iron-chelating activity was
140 observed in these fractions. Thus, fraction F1 showed the highest iron-chelating
141 activity, 4.5-fold higher than that observed in the protein hydrolysate, and 1.8-
142 fold higher than the activity of fractions F2 and F3. However, F1 had a lower
143 histidine content than had F2 and F3, although being very rich in arginine and
144 lysine (24.9 and 11.7 %, respectively). These are polar amino acids that may
145 also be implicated in the high iron-binding capacity observed for this fraction
146 (Berner, & Miller, 1985; Van Campen, 1973).

147 F1, F2 and F3 peptide fractions were further fractioned by gel filtration
148 chromatography in fifteen subfractions. Iron-chelating activity of these
149 subfractions was analyzed and compared with that of the chickpea protein
150 hydrolysate and EDTA (Figure 3). A range of different iron-chelating activities
151 was observed that depends on peptide sizes, amino acid compositions, and
152 sequences of peptides in these subfractions. Subfractions purified from
153 fractions F2 and F3 showed the highest iron-chelating activity, between 11 and
154 17-fold higher than the chelating activity of the protein hydrolysate (Figure 3). In
155 general subfractions with the highest iron-chelating activity also possessed the
156 highest copper-chelating activity (Torres-Fuentes *et al.,* 2011). Thus, in fraction
157 F1, F1D showed the highest iron- and copper-chelating activities. In the same
158 way, subfractions F2C and F2D among F2 subfractions, and F3D and F3E
159 among F3 subfractions, showed the highest iron- and copper-chelating
160 activities. Hence a positive correlation (R^2 = 0.65) was observed between the
161 iron- and copper-chelating activities of all purified subfractions. Small
162 differences in the chelating of Fe or Cu by purified peptides may be atributed to
163 small chemical and physical differences between these two metals, excluding

the oxidation state, that is the same for both. The atomic radius (1.26 Å for iron and 1.28 Å for copper) can be considered as a factor that may also influence their binding to peptides.

Also a positive correlation (R^2 = 0.67), between histidine contents and iron-chelating activity, was observed in purified subfractions (Figure 4A). On the other hand, there was no correlation between the iron-chelating activity and peptide size of these subfractions (Figure 4B). In general, smaller subfractions, below 500 Da (full points, Figure 4B), had higher iron-chelating activities than had subfractions above 500 Da (open points, Figure 4B). Hence, the most active subfractions were F2D, F3D, F2C and F3E. However, F1D showed high activity although its histidine content was lower (8% His and 296 Da). This may be due to the high arginine and lysine contents, as observed in the parent fraction F1. As previously reported for the copper-chelating activity, a combination of high histidine contents, between 20 % and 30 %, and small peptide size provides the best chelating activities.

Cysteine has been recognized as an important amino acid in the generation of iron chelates, especially when derived from meat protein digestion (Glahn et al., 1997; Martínez-Torres, Romano, & Layrisse, 1981; Taylor, Martínez-Torres, Romano, & Layrisse, 1986). However in chickpea-chelating peptides, no correlation was observed between cysteine contents and iron chelation.

In conclusion, previously purified chickpea copper-chelating peptides also showed iron-chelating activity, although iron-chelating activity was higher than copper-chelating activity for the same amounts of peptides assayed. The main determinant in iron-chelating activity was the histidine contents. Hence, the

most active fractions were those with histidine levels above 20 %. The peptide size was not a determinant in the activity although, in general, smaller fractions were more active than were larger fractions. Hence, chelating peptides generated during chickpea protein digestion with pepsin plus pancreatin may be of interest as chelating agents that may improve iron absorption. Thus, these peptides may facilitate the conversion of ferric iron to ferrous, that subsequently enters enterocytes through the DMT1 receptor. Also, peptides with bound iron may enter enterocytes through a peptide transporter localized in the brush border membranes. Iron-chelating activity may also prevent the production of reactive oxygen species, generated in Fenton reactions where iron is implicated. Hence, chickpea iron-chelating peptides may be useful in the food industry to produce fortified food, thus maintaining food quality and appearance. Also, chickpea-chelating peptides may be useful, as well, for the chelation of very important other minerals, in human nutrition, such as calcium or zinc.

Acknowledgments

This work was supported by the Spanish Ministry of Innovation and Science through Research project AGL2007-63580. Thanks are due to María Dolores García-Contreras for technical assistance.

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212 **References**

213 Alaiz, M., Navarro J. L., Girón J., & Vioque, E. (1992). Amino acid analysis
214 by high performance liquid chromatography after derivatization with dietil
215 ethoxymethylenemalonate. *Journal of Chromatography* A, *591*, 181-186.

216 Baynes, R. D., Macfarlane, B. J., Bothwell, T. H., Siegenberg, D., Bezwoda,
217 W. R., Schmidt, U., Lamparelli, R. D., Mayet, F., & Macphail, A. P. (1990). The
218 promitive effect of soy sauce on iron absorption in human subjects. *European*
219 *Journal of Clinical Nutrition*, *44*, 419–424.

220 Beard, J. L. (2001). Iron biology in immune function, muscle metabolism and
221 neuronal functioning. *The Journal of Nutrition*, *131*, 568S-580S.

222 Berner, L. A., & Miller, D.D. (1985). Effects on dietary proteins on iron
223 bioavailability. *Food Chemistry*, *18*, 47-69.

224 Blat, D., Weiner, L., Youdim, M. B. H., & Fridkin, M. (2008). A novel iron-
225 chelating derivate of the neuroprotective peptide NAPVSIPQ shows superior
226 antioxidant and antineurodegenerative capabilities. *Journal of Medicinal*
227 *Chemistry, 51*, 126-134.

228 Carter, P. (1971). Spectrophotometric determination of serum iron at the
229 submicrogram level with a new reagent (ferrozine). *Analytical Biochemistry, 40*,
230 450-458.

231 Chung, Y. C., Chang, C. T., Chao, W. W., Ching-Fwu, L., & Su-Tze, C.
232 (2002). Antioxidative activity and safety of the 50 % ethanolic extract from red

bean fermented by *Bacillus subtilis* IMR-NK 1. *Journal of Agricultural and Food Chemistry, 50*, 2454–2458.

Fidler, M.C., Davidsson, L., Walczyk, T., & Hurrell, R.F. (2003). Iron absorption from fish sauce and soy sauce fortified with sodium iron EDTA. *The American Journal of Clinical Nutrition*, *78*, 274–8.

Glahn, R. P., & Van Campen, D. R. (1997). Iron uptake is enhanced in Caco-2 cell monolayers by cysteine and reduced cysteinyl glycine. *The Journal of Nutrition*, *127*, 642–47.

Lönnerdal, B., Carlsson, J., & Porath, J. (1977). Isolation of lactoferrin from human milk by metal-chelate affinity chromatography. *FEBS Letters*, *75*, 89–92.

Macfarlane, B. J., Riet, W. B., Van Der Riet, W., Bothwell, T. H., Baynes, R.D., Siegenberg, D., Schmidt, U.,Tal, A., Taylor, J. R., & Mayet, F. (1990). Effect of traditional oriental soy products on iron absorption. *The American Journal of Clinical Nutrition*, *51*, 873–880.

Martínez-Torres, C., Romano, E., & Layrisse, M. (1981). Effect of cysteine on iron absorption in man. *The American Journal of Clinical Nutrition, 34*, 322-327.

Murray-Kolb, L. E., Welch, R., Theil, E. C., & Beard, J. L. (2003). Women with low iron stores absorb iron from soybeans. *The American Journal of Clinical Nutrition, 77*, 180–4.

Storcksdieck, S., Bonsmann, G., & Hurrel, R. F. (2007). Iron-binding properties, amino acid composition, and structure of muscle tissue peptides

255 from in vitro digestion of different meat sources. *Journal of Food Science*, *72*,
256 S19–29.

257 Swain, J. H., Tabatabai, L. B., & Reddy, M. B. (2002). Histidine content of
258 low-molecular-weight beef proteins influences nonheme iron bioavailability in
259 Caco-2 cells. *The Journal of Nutrition*, *132*, 245–251.

260 Taylor, P. G., Martínez-Torres, C., Romano, D. E. L., & Layrisse, M. (1986).
261 The effect of cysteine-containing peptides released during meat digestion on
262 iron absorption in humans. *The American Journal of Clinical Nutrition*, *43*, 68-
263 71.

264 Torres-Fuentes, C., Alaiz, M., & Vioque, J. (2011). Affinity purification and
265 characterization of chelating peptides from chickpea protein hydrolysates. *Food*
266 *Chemistry*, *129*, 485-490.

267 Van Campen, D., & Gross, A. (1969). Effect of histidine and certain other
268 amino acids on the absorption of iron-59 by Rats. *The Journal of Nutrition, 99*,
269 68-74.

270 Van Campen, D. (1973). Enhancement on iron absorption from ligated
271 segments of rat intestine by histidine, cysteine, and lysine: effects of removing
272 ionizing groups and of stereoisomerism. *The Journal of Nutrition, 103*, 139-142.

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279 **FIGURE LEGENDS**

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281 **Figure 1** Schematic diagram of the purification process of chickpea iron-
282 chelating peptides

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284 **Figure 2** Iron-chelating activity of (100 g) EDTA, chickpea protein
285 hydrolysate and peptide chelating fractions F1, F2 and F3. Data correspond to
286 the average ± SD of two independent experiments

287

288 **Figure 3** Iron-chelating activity of (30 g) EDTA, chickpea protein
289 hydrolysate and chelating peptide fractions purified by gel filtration
290 chromatography. Data correspond to the average ± SD of two independent
291 experiments

292

293 **Figure 4** Correlation of iron-chelating activity of chickpea-chelating
294 peptide subfractions with their His content **(A)** and peptide size **(B)**

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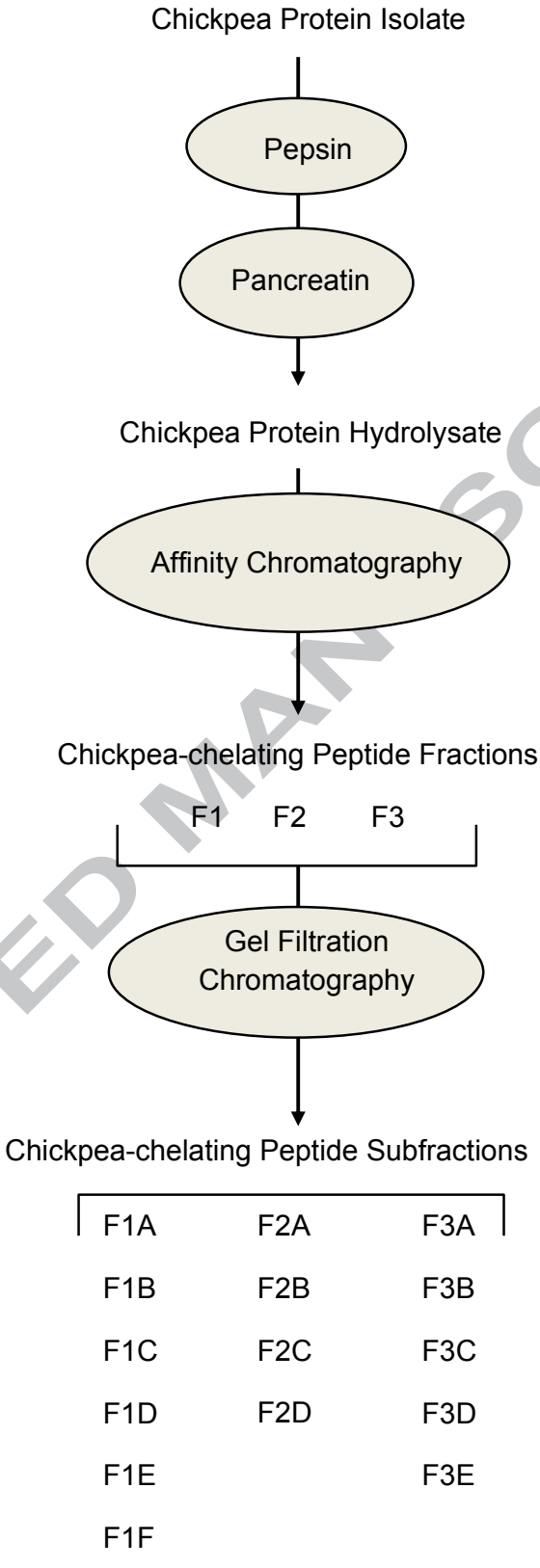

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Figure 1

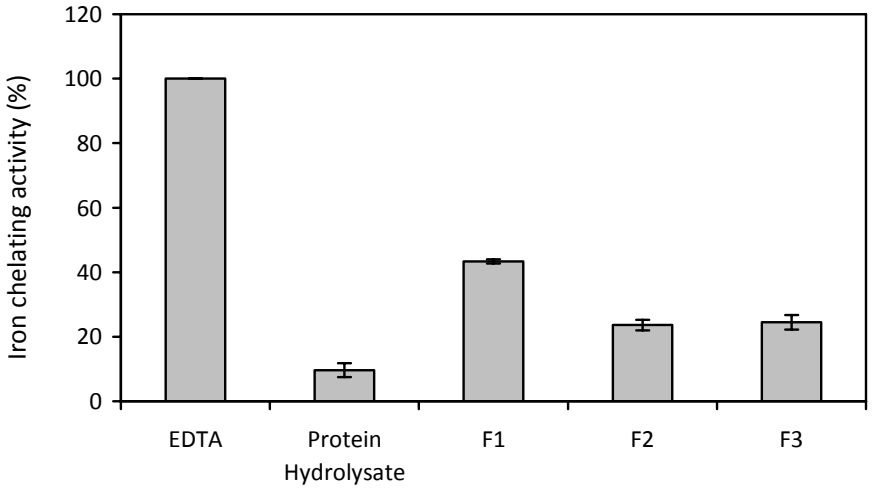


Figure 2

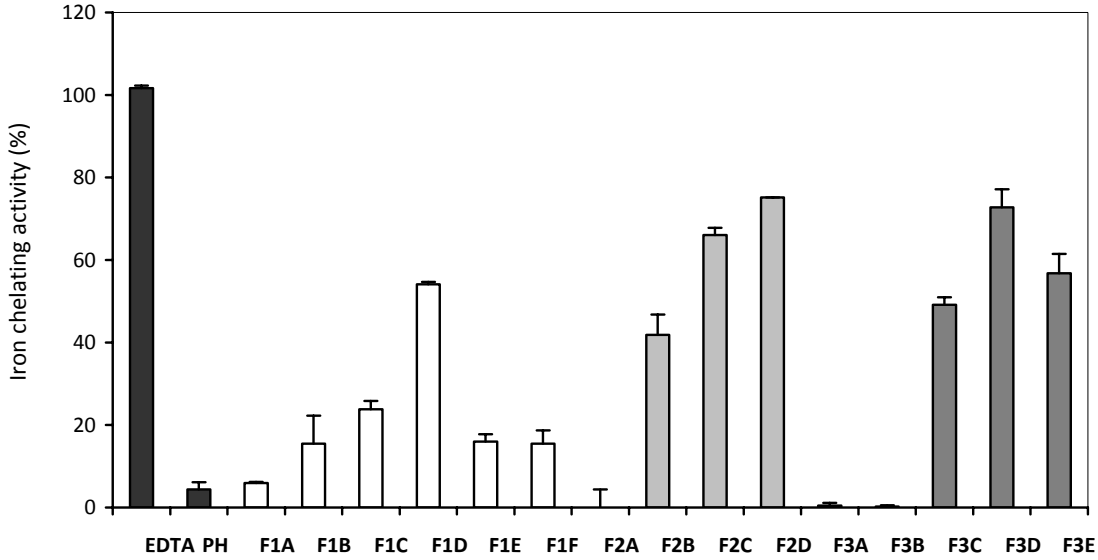
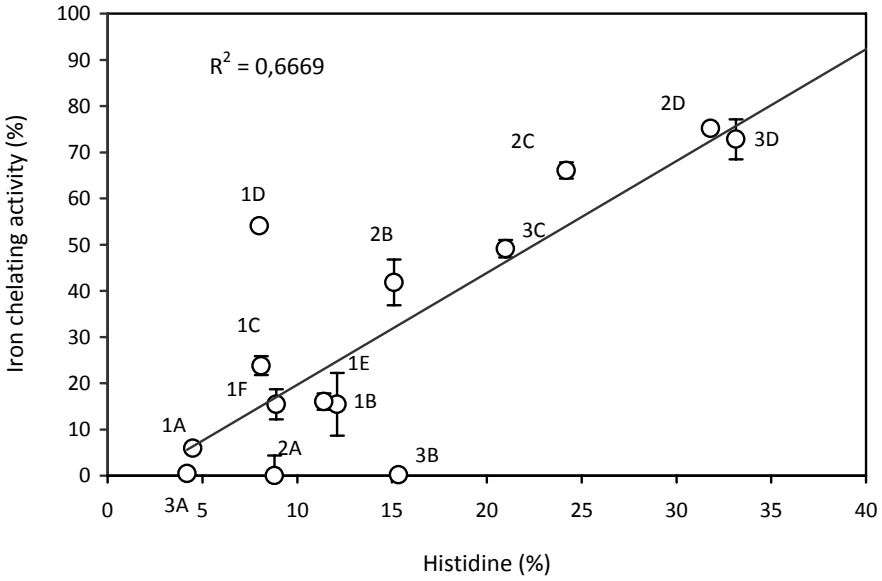


Figure 3



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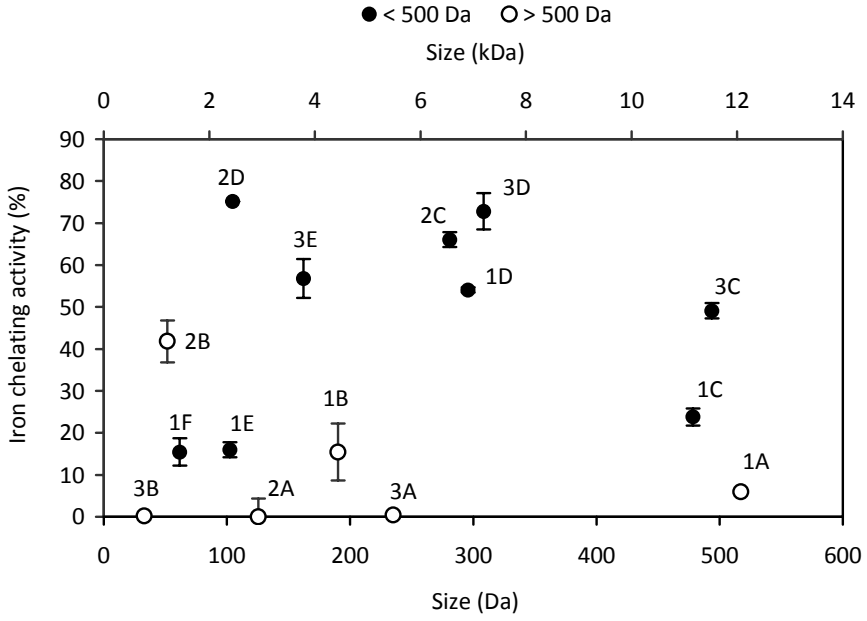

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329 Figure 4

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331 Highlights.

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334 Chickpea protein hydrolysates produced with pepsin and pancreatin contain
335 iron chelating peptides > A positive correlation between histidine contents and iron
336 chelating activity was observed > These peptides may be useful in increasing iron
337 solubility and bioavailability.

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