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In vitro study of the inhibitory effect of Fe(II), Fe(III) and Zn(II) ions on the activity of trypsin

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Abstract

The effect of Fe(II), Fe(III) and Zn(II) addition on the in vitro digestibility of casein was examined using trypsin as the proteolytic enzyme. The in vitro digestibility was determined by change in trichloroacetic acid (TCA) solubility of casein and by potentiometric titration. The effect of all three metal ions was also examined on the digestion of casein heated at 80°C for 15 min prior to the enzymic hydrolysis. Heat treatments did not cause significant changes in the proteolysis of casein. The addition of Fe(II) and Fe(III) clearly slowed the proteolysis in both heated and unheated casein. The decrease in rate of proteolysis was in a direct linear relationship with the amount of Fe(II) and Fe(III) addition. The inhibitory effect of Fe(III) was more pronounced as compared to Fe(II). The addition of Zn(II) did not affect the proteolytic activity in both heated and unheated casein.

Keywords: Casein; In vitro digestibility; Trypsin; TCA; Potentiometric titration

1. Introduction

The turning of proteins into amino acids is the responsibility of a group of hydrolytic enzymes, the proteolytic enzymes. Pepsin, trypsin, chymotrypsin and amino peptidases are responsible for hydrolysis of ingested proteins to amino acids in the digestive tract of man. The proteolytic enzymes are also by far the most important group of enzymes in the food processing industry. These are used in the production of cheeses, chill-proofing of beer, tenderization of meat and modification of the properties of the proteins of cereals in bread and cereal manufacture.

Many factors can have an effect on the digestibility of proteins (Timofeeva, Lezvitova, Egorova, Nikitina & Starchenkov, 1995) . The crosslinking and racemization reactions caused by heating and/or alkali treatments produce abnormal amino acids, such as lysinoalanine (LAL) and D-amino acids, which the body cannot utilize as effectively as normal amino acids (Friedman, Gumbmann & Masters, 1984).

Variation in pH and ionic strength has also been found to impair the digestibility of proteins (Pedersen & Eggum, 1983; Rothenbuhler & Kinsella, 1985). Similarly the presence of various metal ions can cause changes in the hydrolysis of proteins (Beyer, Kirchgessner & Steinhart, 1975, 1976; Kirchgessner, Roth & Roth-Maier, 1974, Kirchgessner, Steinhart & Weininger-Rusemeyer, 1980; Wieninger-Rustemeyer, Kirchgessner & Steinhart, 1981).

The alteration in hydrolysis of proteins due to metal ions is based on the complex forming ability of protein or enzyme with metal ions (Gresh & Geissneer-Prette, 1997; Kesissoglou, 1995; Solomon, Lowry, Root & Hemming, 1995; Van Wart & Harold, 1996; Yoshino, Murakami, & Kawano, 1998). Protein or enzyme molecule possessing functional groups like ketones, amides, amines and phenols have strong affinity for metals. In addition to this, metallic ions particularly alkaline earth metals and transition metals show a marked tendency to form complexes. Due to the presence of incompletely filled d orbitals, transition metals act as Lewis acids and therefore react with Lewis bases, usually called ligands to form complexes (Gresh & Geissneer-Prette, 1997; Kesissoglou, 1995; Solomon et al., 1995; Van Wart & Harold, 1996). Among alkaline earth metals particularly

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Mg(II), Ca(II) show appreciable tendency to form complexes with ligands which have oxygen as donor atoms.

The purpose of this study was to examine the effect of metals on enzymic digestion of proteins, if any, due to complex formation between the metal and protein or between the metal and enzyme.

The effect of Fe(II), Fe(III) and Zn(II) addition was studied using casein as the substrate and trypsin as the proteolytic enzyme. Although many other metal ions have a tendency to form complexes with proteins, the reason for selecting Fe(II), Fe(III) and Zn(II) for the study was the temptation to deduce from the available data that iron is abundant in the diet and its deficiency should be rare but is poorly absorbed from the intestine, partly due to the formation of insoluble Fe(III) hydroxide at pH 8 in the duodenum and partly due to the degree of absorption being dependent on the quantity and nature of other compounds in the diet. Likewise the potential for Zn(II) absorption to be reduced by the presence of proteins in the diet has not been realised, though it is an essential component of the active site of many enzymes.

2. Materials and methods

In vitro digestibility of casein was determined by change in tricholoroacetic acid (TCA) solubility of casein and by potentiometric titration.

2.1. Change in the TCA solubility of casein

A solution of 3 mg/ml of casein was prepared by dissolving 0.3 g casein (22086, Fluka [9000-71-9]) in 100 ml of 0.01 M phosphate buffer (pH 7.5). To six 15-ml pyrex centrifuge tubes, each containing 1 ml of the casein solution, 0.0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ml of 0.01mg/ml Fe(II) solution (prepared by dissolving 0.0702 g $FeSO_4$ $(NH_4)_2$ $SO_4.6H_2O$ in 1 l of water) was added. The volume in each tube was brought to 2 ml with 0.01M phosphate buffer (pH 7.5) and the tubes were placed in water bath at 37°C. 0.1 ml of the 0.2 mg/ml trypsin solution (prepared by dissolving 20 mg of trypsin, EC.3.4.21.4, 4019 Sigma [9002-07-7] in 100 ml of 0.01 M phosphate buffer, pH 7.5) was added to the first tube, and a stop watch was started. Each subsequent tube received 0.1 ml of trypsin at 30-s intervals. Three millilitres of 5% TCA (91228, Fluka [76-03-9]) was added in the same order to each tube exactly 20 min after the addition of casein. The contents of the tubes were mixed well. The tubes were removed from the water bath, allowed to stand for 1 h and centrifuged at 8000 r.p.m. for 5 min. The absorbance of the supernatants was read at 280 nm on UV spectrophotometer (Shimadzu 160A). The readings were corrected for the values of blanks. The blank of control was prepared by mixing 1 ml casein and 3 ml of TCA while the blank for 1–5 tubes was prepared by mixing 1 ml casein, 3 ml TCA and 0.1 ml trypsin.

The effect of adding Fe(III) and Zn(II) was examined by the same procedure and at the same pH 7.5. The effect of all three metal ions was also examined in heated samples of casein. Heat treatments were carried out at 80°C for 15 min prior to hydrolysis.

2.2. Potentiometric titration

Eight millilitres of 0.005 M Tris/HCl buffer, pH 7.9, and 1 ml of casein were added to a small beaker. The beaker was placed on a magnetic stirrer and the electrode of pH meter (CG 825, Schott) was introduced into the liquid. With the aid of 10 ml burette, 0.1 N NaOH was added to adjust the pH to 8.0. Trypsin solution (1 ml) was then added. The pH of the reaction mixture decreased. The stop watch was started when the pH of 7.9 was reached. At that time, approximately 0.01 ml of 0.1 N NaOH was added and the time at which the pH 7.9 was again reached was recorded. After five or six repetitions, the volume of NaOH added was plotted versus time.

The effect of adding Fe(II), Fe(III) and Zn(II) was examined by adding 0.1–0.5 mg of the metal ion to casein before addition of trypsin and then adding 0.1 N NaOH to pH 8.0. This method was also applied to both heated and unheated samples of casein. Heat treatment was given at 80°C for 15 min before hydrolysis.

3. Results and discussion

Heat treatments at 80°C for 15, 30 and 60 min did not cause significant changes in the proteolysis of casein. On the other hand, the addition of Fe(II) and Fe(III) with a short heat treatment (15 min at 80°C) clearly slowed the proteolytic reaction carried out under the same conditions. The change was in a direct linear relationship with the amount of iron addition (Figs. 1–3). The addition of Zn(II) did not cause any significant change (Figs. 1 and 4). The same effect was observed in the case of unheated casein.

The above observations suggested that the inhibitory effect of iron on proteolysis was linked more with its effect on trypsin than with changes in substrate protein composition caused by the iron addition, and the resultant changes in digestibility.

In addition to this study, the inhibitory effect of iron on proteolytic enzymes had been observed e.g. in vitro pepsin digestion of soya protein and hemoglobin (Beyer et al., 1975; Steinhart, Beyer & Kirschgessner, 1975). According to the studies, the inhibitory effect of iron must be connected with the formation of the complex between proteolytic enzyme and iron and not between protein and iron. At the acidic pH ranges (e.g. pH 2.2) needed in pepsin proteolysis, no complex was formed between substrate protein and the metal ions. The substrate

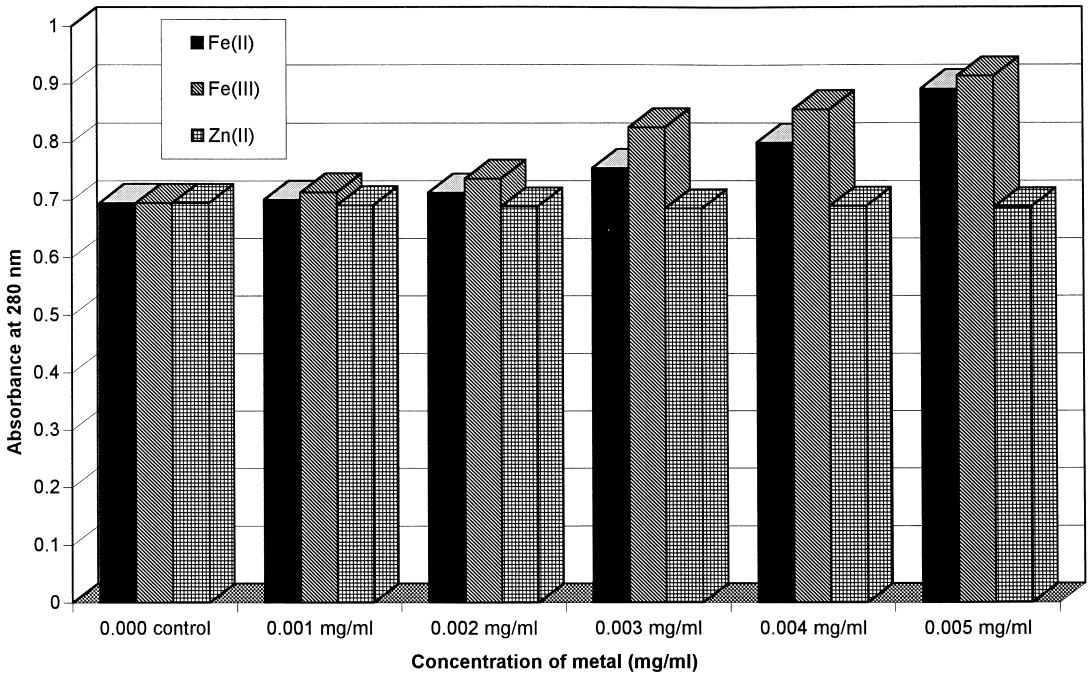

Fig. 1. Effect of adding metal ion on the rate of proteolysis of casein heated at 80°C for 15 min. Rate of proteolysis measured as change in trichloroacetic acid solubility.

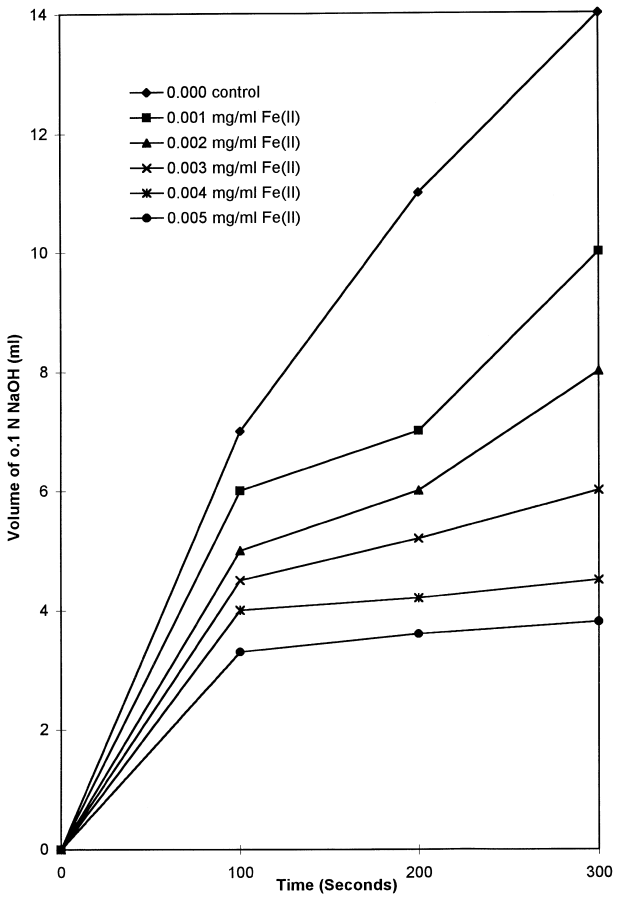

Fig. 2. Effect of adding Fe(II) on the rate of proteolysis of casein heated at 80°C for 15 min. Rate of proteolysis measured as volume of 0.1 N NaOH consumed with respect to time.

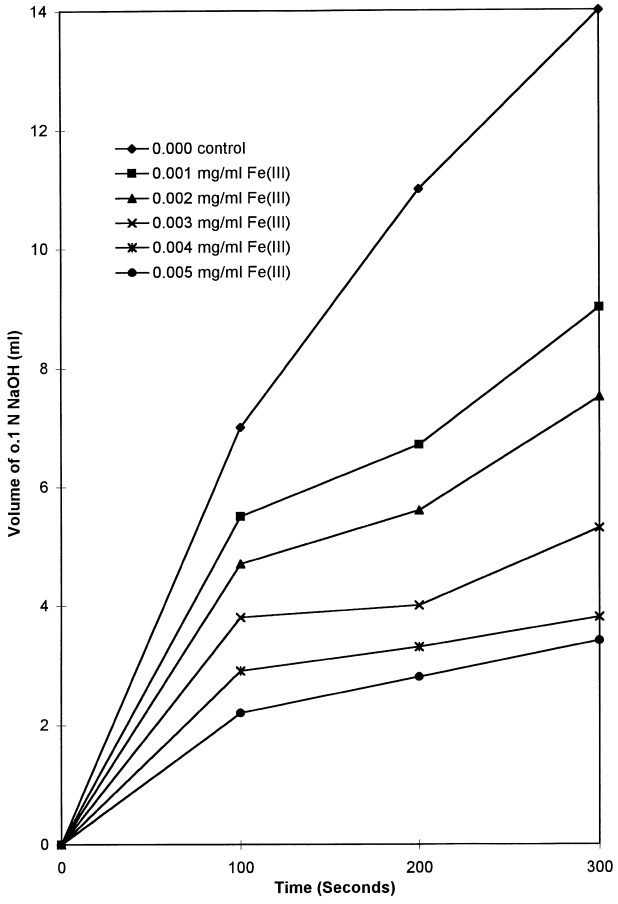

Fig. 3. Effect of adding Fe(III) on the rate of proteolysis of casein heated at 80°C for 15 min. Rate of proteolysis measured as volume of 0.1 N NaOH consumed with respect to time.

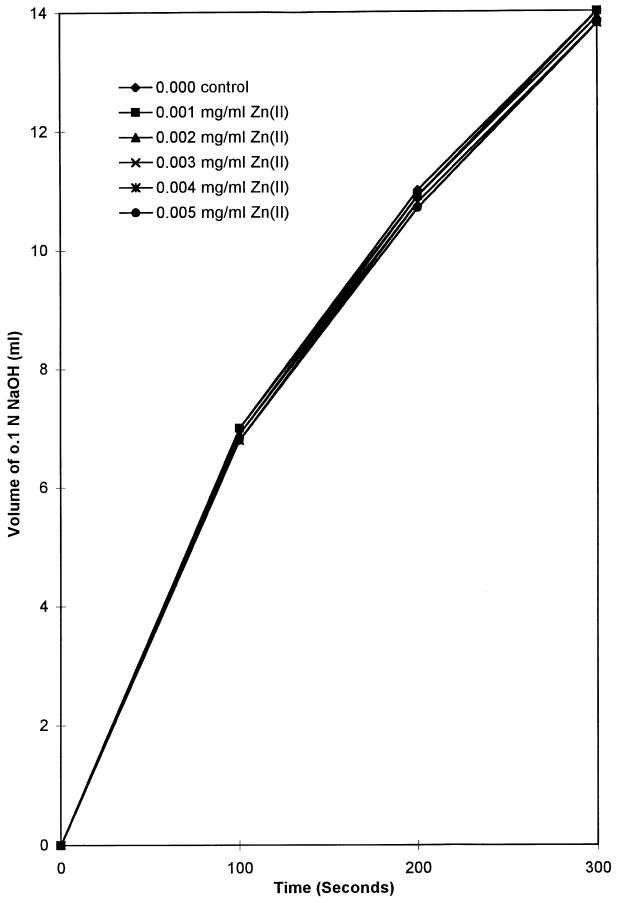

Fig. 4. Effect of adding Zn(II) on the rate of proteolysis measured as volume of 0.1 N NaOH consumed with respect to time.

protein in these experiments was soya protein and the metal ion examined was Cu(II).

As compared to Zn(II), iron formed very stable bonds with proteolytic enzymes, because the stability constant of iron in the various complex compounds had been found to be higher than that of other microelements (Beyer et al., 1976). Connected with the observed higher stability constants of Fe(III) complex compounds, Fe(III) salts had also been shown to have a greater inhibitory effect than Fe(II) salts (Beyer et al., 1976).

4. Conclusions

The amounts of iron addition which clearly inhibited the proteolytic enzyme activity in this study were roughly the same as the amounts found in foods enriched with iron. In view of the central role played by iron-enriched foods in the nutrition of infants and children in particular, the importance of iron as a factor possibly affecting the utilization of proteins would be clarified by studies carried out in vivo conditions. As it is possible that a strong complex was formed between iron and protein, diminished availability of iron from food containing protein may also have significance.

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