In Vitro Digestibility of Plastein Gel Prepared from Peptic Digest of Casein

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ABSTRACT

In vitro digestibility of plastein prepared from casein hydrolyzate was evaluated using proteolytic enzymes. Pancreatin was the most effective digestion enzyme, followed by trypsin, α-chymotrypsin, and pepsin was the least effective. The in vitro digestibility of plastein was higher than that of casein. Amino acid analysis of plastein showed that plastein was rich in glycine, leucine, methionine, and histidine. The amino acid pattern of plastein was remarkably similar to FAO/WHO/UNU standard for amino acid requirements.

INTRODUCTION

Plastein formation was discovered more than 100 years ago. It is formed from the interaction among peptides found in the protein hydrolyzate through covalent or noncovalent bonding by proteolytic action. The product, plastein, is a gel-like proposed by many scientists for food uses since it has many advantages (Eriksen and Fagerson, 1978; Belikov and Gololobov, 1982; Watanabe and Arai, 1988). Among the information of plastein, little has been found about its nutritional quality. Some workers looked at the amino acid composition of plastein and made a comparison with FAO/WHO suggested patterns (Onoue and Riddle, 1973; Arai *et al.*, 1976) or measured the chemical score of the amino acids (Yamashita *et al.*, 1970). However, there is little information on the digestibility of plastein. Although the amino acid composition of protein is most important in the evaluation of its nutritive quality, it should also be accompanied by an evaluation of its digestibility since it is useful for the determination of the available amino acids. The digestibility of protein may be obtained by using rats bioassay or by *in vitro* digestion. The *in vivo* method for measurement of protein digestibility using rats or other animals is expensive and time consuming procedure, therefore *in vitro* methods were developed by many workers (Buchanon, 1969; Buchanon and Byer, 1969; Saunders *et al.*, 1973; Hsu *et al.*, 1977). The *in vitro* method use proteolytic enzymes or microorganisms for the measurement of protein quality. The enzyme pepsin, trypsin, chymotrypsin, papain and pancreatin or their combinations were widely used. Some workers made a correlation between *in vivo* and *in vitro* methods in order to find the relationship of those two methods (Buchanon, 1969; Hsue *et al.*, 1977).

The objective of the study was to evaluate the digestibility of plastein prepared from peptic casein hydrolyzate using proteolytic enzymes as a tool.

MATERIALS AND METHODS

Casein and proteinases were purchased from Sigma Chemical Co., U.K. and used through out the study without any additional treatment or purification. The purity of the enzymes are expressed as their protein content analyzed by absolute method as described by Whitaker and Granum (1980). The proteinases include pepsin, trypsin, α-chymotrypsin, and pancreatin. All chemicals used in this experiment were laboratory grade reagents supplied by The British Drug House (BDH) Ltd. England, Sigma Chemical Co. Ltd. England, Hopkin & William (HW) Ltd. England, Fision Scientific Apparatus (FSA) Ltd. England, May and Baker England, and the Merck West Germany or otherwise stated.

Preparation of the Substrate for Plastein

Casein was hydrolyzed with pepsin (Sigma Chemical Co.) under the following procedures. Casein was suspended in dilute HCl to give a concentration of 1% w/v. The pH was adjusted to 1.8 by 4M HCl solution. The casein suspension was prewarmed at 37°C for 10 min, then pepsin was added to give an enzyme-substrate ratio of 1:100. The reaction mixture was incubated at 37°C for 24 hours in a water bath shaker. The hydrolysis of protein was stopped by increasing pH to 7.0 with 1M NaOH solution. Thereafter, it was centrifuged at 10,000 × *g* for 15 minutes. The pellets were discharged and the supernatant was subjected to ultrafiltration apparatus using 10,000 molelecular weight cutoff membrane cellulose. The filtrate was then freeze dried and used for substrate for plastein.

Plastein Synthesis

The plastein synthesis was carried out with α-chymotrypsin. Freeze dried casein hydrolyzates were dissolved in 0.1M citrate phosphate buffer pH 5.6 to give a substrate concentration of 30% w/v. The α-chymotrypsin was then added to the substrate solution in order to 1/100 (w/v) ratio. The mixture was the incubated at 37°C for 48 hours. The synthesis of plastein was stopped by dilution to 10 fold with the same buffer mentioned above and centrifuged at 2,000 × *g* for 20 minutes. The supernatant was discarded and the pellets (plastein) were freeze dried.

***In Vitro* Digestibility**

The extent of digestibility of plastein with proteases were determined using enzyme to substrate ratio of 1:100 w/w in a final volume 50 ml and assay time at 24 hours in each case with 1% w/v substrate concentration. A number of proteases, *i.e.*, pepsin, trypsin, α-chymotrypsin, and pancreatin were used to evaluate *in vitro* digestibility of plastein. For the assay of pepsin, the plastein was suspended in 0.1M KCl-HCl buffer pH 1.8, while for trypsin, chymotrypsin, and pancreatin digestion the plastein was suspended in 0.1 M borate buffer pH 8.0. The assay was conducted in a water bath with mild shaking. Casein was used as a standard enzymic digestibility. The degree of proteolysis was determined by TNBS method (Goldfarb, 1966; Kuchro *et al.*, 1983; Ihekeronye, 1986).

Amino Acid Composition

The amino acid composition of plastein was analyzed by HPLC technique after 24 hours hydrolysis of plastein with 6M HCl under nitrogen gas at 110°C. The procedure was based on the method of Lookhart and Jones (1985). Since tryptophan is destroyed during hydrolysis (Anglemier, 1978; Armstrong, 1989) and proline is not covered by this method (Lookhart and Jones, 1985) they were analysed separately. Tryptophan was determined using the method of Miller (1967), while proline was determined spectrophotometrically by the method of Chinard (1952).

Measurement of the Activity of Enzymes

The procedure of Bergmeyer (1974) was used to determine the proteinases activity. Pepsin activity was assayed as follows. The substrate haemoglobin (2g) was dissolved in 100 ml of 0.06 M hydrochloric solution, centrifuged at 4.000 × *g* for 15 minutes to remove insoluble materials. To the substrate solution (2.5 ml) which had been equilibrated at 35°C was added 0.5 ml prewarmed of pepsin solution (1 mg/ml 0.006 M HCl), mixed gently and incubated for exactly 10 minutes. The reaction was terminated by the addition of 5.0 ml of 5.0% w/v trichloroacetic acid (TCA) solution. After standing for several minutes at the same temperature of incubation, the mixture was filtered through a Whatman No. 4 filter paper and TCA-filtrate was collected. The absorbance of the filtrate was read at 280 nm using spectrophotometer (Perkin Elmer Ltd., USA, Model 552). The specific activity of pepsin is expressed in pepsin unit (PU). One pepsin unit, as difined by Anson (Bergmeyer, 1974) is the amount of enzyme which hydrolyzes 0.1 g haemoglobin giving an increase in absorbance of 0.01 min-1 at 280 nm under condition used.

The activity of trypsin, chymotrypsin, and pancreatin are expressed in caseinolytic activity. The substrate solution was prepared by suspending 1.0 g hammarstein casein (BDH Ltd. England) in 95 ml 0.1 M borate buffer pH 8.0. After heating in a boiling water bath for about 10 minutes, or until casein was dissolved, 1.1 ml of 5% w/v calcium chloride solution

was added and the volume was made up to 100 ml with buffer. A 2.0 ml aliquot of substrate solution was equilibrated at 35°C, then 2 ml of freshly prepared enzymes solution was added, mixed and incubated for exactly 20 min. Trichloroacetic acid solution (5 ml, 5% w/v) was then added to stop the reaction. After standing for 30 minutes at 35°C the mixture was filtered through a Whatman No. 4 filter paper and the filtrate was collected. The absorbance of the filtrate was measured at 280 nm using Perkin Elmer 552 spectrophotometer. A unit activity is the amount of enzyme which hydrolyzes 0.01 *g* casein giving an increase in the absorbance of 0.01 min-1 under condition used. The unit activity of the enzymes were calculated according to the formula as indicated by Bergmeyer (1974).

RESULTS AND DISCUSSION

The degree of proteolysis of plastein and casein was measured at appropriate periods of time using the TNBS method during the incubation time. The result is showed in Figure 1, 2, 3, and Figure 4. In general, subyect the proteolytic enzymes to protein solution would increased the degree of hydrolysis, *i.e.* increase in amino groups detected by trinitrobenzenesulfunic acid (TNBS). However, there were defferences in the result due to the defferences of the activity and kind of enzymes. As noted by Alder-Nissen (1976; 1986), among the important factors in the hydrolysis of protein are the type of enzyme and pH value used. The degree of hydrolysis were also not related to the activity of enzymes (see Table 1). High activity was found for the pepsin followed by chymotrypsin, pancreatin, and trypsin. As can be seen from Figure 1 to Figure 4, among the proteinases used, pancreatin is most effective to digest the plastein. Pancreatin is a mixture of enzymes mainly trypsin, chymotrypsin and pepsin. As a multi enzyme preparation, pancreatin is less likely to be affected by a specific inhibitors leading to the rapid and effective digestion (Hsu *et al.*, 1977). Some workers showed that pancreatin is useful for evaluating the *in vitro* protein digestibility for foods products (Saunders *et al.*, 1973; Hsu *et al.*, 1977; Jenkins and Emmons, 1983). Using pancreatin the degree of proteolysis of plastein found in the present study was around 60% after 24 hours digestion (Figure 4), while using trypsin and chymotrypsin the degree of proteolysis was only 25 – 30% (Figure 3). Pepsin is a less effective enzyme for digesting plastein (Figure 1). This is not the case for protein digestion by pepsin since pepsin is usually a good proteolytic enzyme and widely used for evaluation of *in vitro* protein digestibility (Saunders *et al.*, 1973). It is that the structure of plastein does not available for the activity of pepsin. However, compared to the intact protein, *i.e.* casein, the *in vitro* digestibility of plastein was higher. Casein is widely used as standard evaluation for protein quality by most bioassay method. This milk protein has high biological value and thus more digestible than other proteins.

Table 1. **Protein content and the activity of various proteinases**

Proteinase	Protein content*) (%)	Specific activity (U/mg enz/ml)
Pepsin	72.85	19.687
α-Chymotrypsin	87.45	5.635
Pancreatin	57.65	3.620
Trypsin	72.54	1.189

*) Determine by absolute method (Whitaker and Granum, 1980)

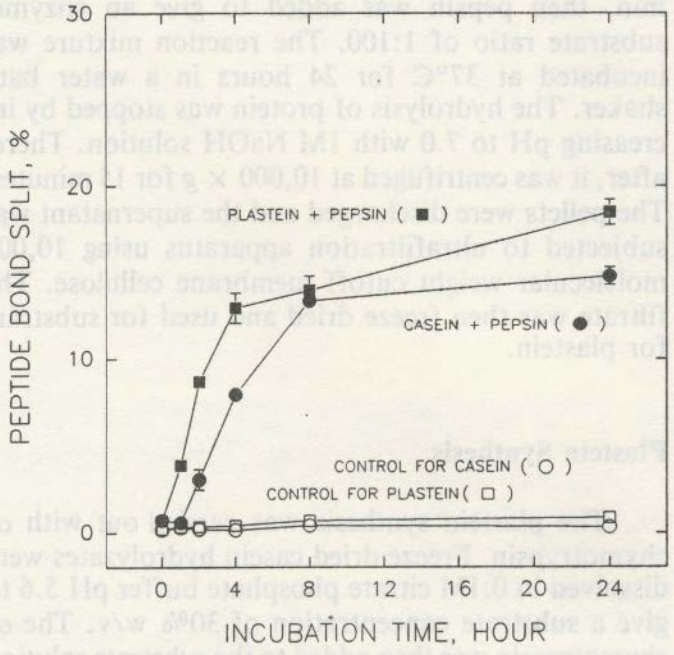

Figure 1. **In vitro digestibility of plastein and casein by pepsin**

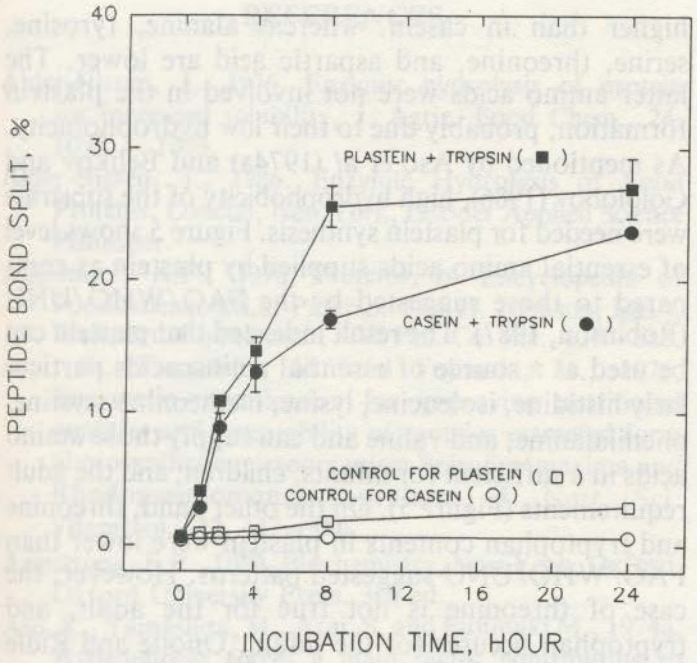

Figure 2. **In vitro digestibility of plastein and casein by trypsin**

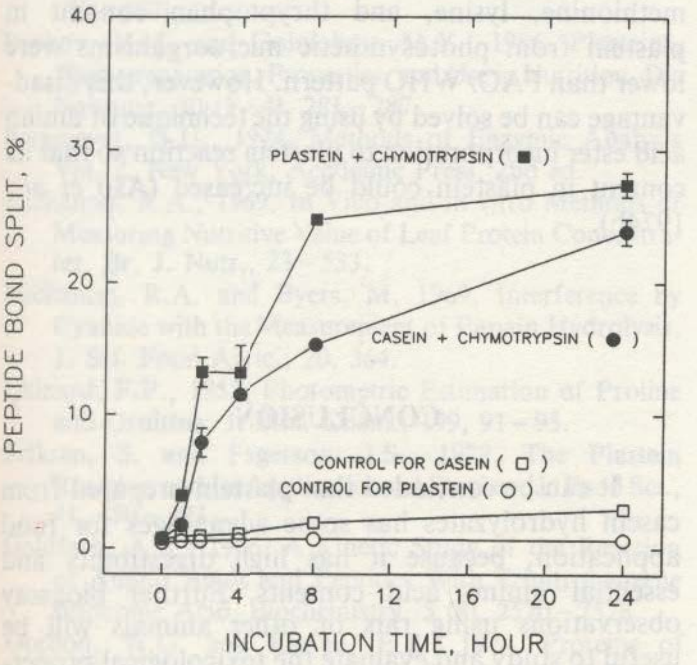

Figure 3. **In vitro digestibility of plastein and casein by chymotrypsin**

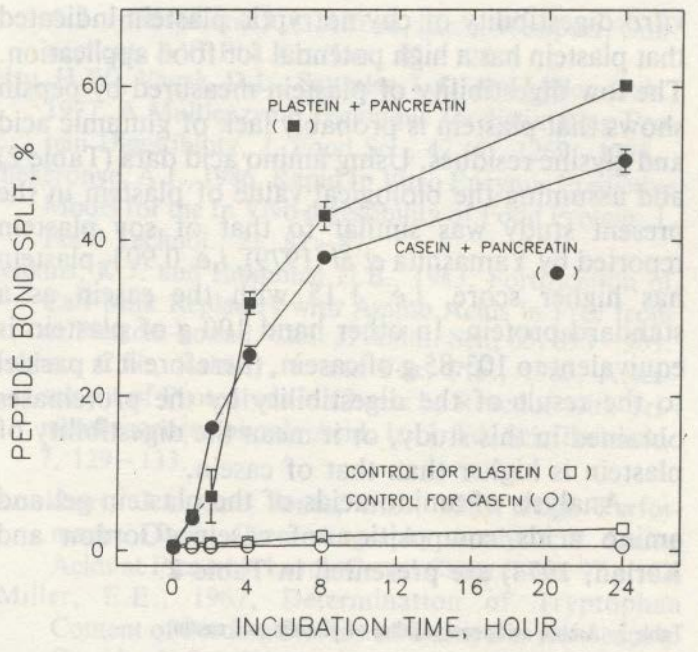

Figure 4. **In vitro digestibility of plastein and casein by pancreatin**

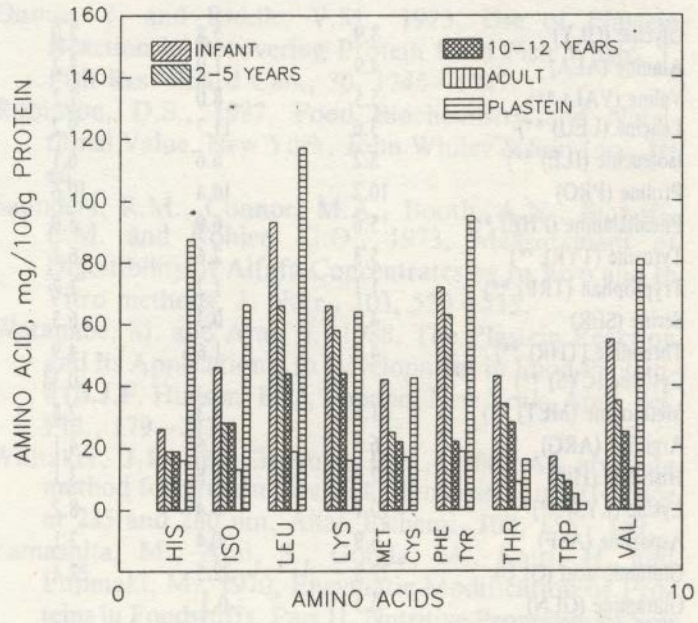

Figure 5. **Essential amino acid patterns of plastein and the 1985's FAO/WHO/UNU suggested patterns of human amino acid requirements**

Noack and Hajos (1984) in their experiment found that digestibility of the methionine-enriched plastein was similar to that of the intact casein. Yamashita *et al.* (1970) evaluated the bio-utilization of soy plastein using rats and found that the true digestibility of plastein was 90,3% and the biological value was 66.8. On the other hand, the digestibility of casein is around 94 – 97% using rats (Robinson, 1987). The high *in*

vitro digestibility of chymotryptic plastein indicated that plastein has a high potential for food application. The low digestibility of plastein measured by pepsin shows that plastein is probably lack of glutamic acid and glysine residues. Using amino acid data (Table 2) and assuming the biological value of plastein in the present study was similar to that of soy plastein reported by Yamashita *et al* (1979), *i.e.* 0.903, plastein has higher score, *i.e.* 1.15 with the casein as a standard protein. In other hand 100 g of plastein is equivalent to 103.85 g of casein, therefore it is paralel to the result of the digestibility by the proteinases obtained in this study, or it mean the digestibility of plastein is higher than that of casein.

Analysis of amino acids of the plastein gel and amino acids composition of casein (Gordon and Karlan, 1974) are presented in Table 2.

Table 2. **Amino acids composition of plastein and casein**

Amino acid (g/100 g protein)	Casein hydrolyzate	Plastein	Casein *)
Glycine (GLY)	3.9	5.8	2.0
Alanine (ALA)	4.9	1.9	3.2
Valine (VAL) **)	5.5	8.0	7.2
Leucine (LEU) **)	3.6	11.7	9.2
Isoleucine (ILE) **)	5.2	6.6	6.1
Proline (PRO)	10.2	10.4	10.6
Phenilalanine (PHE) **)	5.6	6.9	5.0
Tyrosine (TYR) **)	5.3	2.6	6.3
Tryptophan (TRP) **)	1.1	1.2	1.7
Serine (SER)	4.1	0.3	6.3
Threonine (THR) **)	4.4	1.6	4.9
Cysteine (CYS) **)			0.34
Methionine (MET) **)	11.1	4.3	2.8
Arginine (ARG)	6.5	3.6	4.1
Histidine (HIS)	2.4	8.8	3.1
Lysine (LYS) **)	10.1	6.4	8.2
Aspartine (ASP)	6.9	0.4	7.1
Glutamic acid (GLU)	22.8	22.5	22.4
Glutamine (GLN)	0.3	0.3	
Asparagine (ASN)	1.1	1.3	

*) Adapted from W.G. Gordon and E.B. Karlan, 1974.
**) Essential amino acids (average from three replicates)

It can be seen that the plastein has the same amino acids as casein. This mean that all amino acids found in the protein used for preparation of substrate for plastein are involved in the plastein formation. Glycine, leucine, methionine and histidine found in plastein are higher than in casein, whereas alanine, tyrosine, serine, threonine, and aspartic acid are lower. The latter amino acids were not involved in the plastein formation, probably due to their low hydrophobicity. As mentioned by Aso *et al* (1974a) and Belikov and Gololobov (1986), high hydrophobicity of the substrate were needed for plastein synthesis. Figure 5 shows level of essential amino acids supplied by plastein as compared to those suggested by the FAO/WHO/UNU (Robinson, 1987). The result indicated that plastein can be used as a source of essential amino acids particularly histidine, isoleucine, lysine, metheonine, cystine, phenilalanine, and valine and can supply those amino acids in a sufficient for infants, children, and the adult requirements (Figure 5). On the other hand, threonine and tryptophan contents in plastein were lower than FAO/WHO/UNU suggested patterns. However, the case of threonine is not true for the adult, and tryptophan occures for the infant. Onoue and Ridle (1973) also found that all essential amino acids in plastein prepared from fish waste except tryptophan are significantly in excess over the FAO patterns of amino acids, whereas Arai *et al*. (1976) claimed that methionine, lysine, and thryptophan content in plastein from photosynthetic microorganisms were lower than FAO/WHO pattern. However, this disadvantage can be solved by using the technique of amino acid ester incorporation to plastein reaction so that its content in plastein could be increased (Aso *et al*., 1974b).

CONCLUSION

It can be concluded that plastein prepared from casein hydrolyzates has some advantages for food application, because it has high digestibility and essential amino acid contents. Further bioassay observations using rats or other animals will be useful to study and evaluate the toxicological properties of plastein.

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