

Approaches to the Use of Plastein Reaction in Oily Fish

—Preparation and Characterization of Plastein Products—

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Abstract

Plastein reaction was applied to water soluble protein and waste material of oily fishes (sardine, *Sardinops melanosticta*). Formed products were treated with ethanol and normal hexane then dried. Chromatographic behavior, lipids content, total protein content, protein digestibility, solubility, hydration capacity, thiobarbituric number and amino acid content of dried products were studied. Sephadex chromatography of end products and hydrolyzed solution showed only a slight change in molecular weight distribution. Dried products had high protein content, low lipid content (about 0.3–0.5%), nice protein digestibility (75%), low thiobarbituric number and high proportion content of essential amino acids. Protein recovery was 67.5% for water soluble protein and 28% for waste protein. It was possible to get gel like products.

An underutilized resource of protein is present in water soluble protein and waste material of oily fishes, but the main problem is to develop adequate technique for the recovery of this resource. No work have been reported until now in the use of plastein reaction in water soluble protein and waste protein of oily fishes.

Plastein reaction involved the enzymatic hydrolysis of proteins into small molecular polypeptides and the rearrangement of these peptides, into new protein-like substance, by transpeptidation, noncovalent bonds, hydrophobic interaction¹⁾ or condensation reactions²⁾, using enzyme (protease) as a driving force.

One problem related with the use of enzyme in food industries is the cost of the enzyme. In this report were used two microbial proteases Molsin (from *Aspergillus saitoi*) and Biopraxe (from *Bacillus subtilis*), which have been reported by some authors to present a nice plastein productivity³⁾. And also microbial proteases are cheaper than animal or plant proteases.

The present study was done to determine the feasibility of utilize plastein reaction in water soluble protein and waste material of oily fishes, and evaluate the characteristics of formed products. Gel chromatography was also performed to determine, the molecular weight distribution of plastein products and hydrolyzed solutions.

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Materials and Methods

Methods of Evaluation.

Protein determination were based on Kjeldahl nitrogen $\times 6.25$, moisture was determined by heating to a constant weight in a drying oven at 135°C for 2 hours as described by AOAC⁴⁾. The method of Bligh and Dyer⁵⁾ was used for total lipids. Amino acids were analyzed with a Hitachi automatic amino acid analyzer, model 034, as described by MOORE et al.⁶⁾

Tryptophan was determined by alkaline hydrolysis procedure, as described by NOLTMAN et al.⁷⁾ Cysteine was analyzed by performic acid oxidation method as described by MOORE et al.⁸⁾

Rancidity was determined using the 2-Thiobarbituric acid method (TBA), as described by SHIBATA et al.⁹⁾ Results were expressed in TBA number according to SINNHUBER et al.¹⁰⁾

In vitro digestibility was determined as described by Hsu et al.¹¹⁾ A multienzyme system was added to protein suspension at pH 8.0 and the drop in pH was recorded for 10 min. Merk Hammarsten casein was used as standard substrate.

At first was used pepsin as described by OSHIMA¹²⁾, but the results were in agreement with multienzyme method.

Solubility profile of plastein products was determined, dissolving 1 g of dried product in solutions at various pH, from pH 2 to 12, then holding the samples at the above mentioned pH for 2 hours and centrifuged at 5000 rpm for 15 min.

Protein contents in supernatant and precipitate were determined by Biuret reaction as described by OOSHIRO.^{13,14)}

$$\% \text{ Solubility} = \frac{\text{mg of protein in supernatant}}{\text{mg of total protein}} \times 100$$

Molecular weight distribution was determined by gel chromatography as described by HOFSTEN et al.¹⁾, using Sephadex G-50 equilibrated with 50% v/v acetic acid and eluted with the same solvent. Fractions of constant volume, usually 6.4 ml, were collected and the molecular weight distribution of plastein products and hydrolyzed solutions, was determined in the eluates measuring the absorbance at 280 nm.

Degree of hydrolysis was determined as described by YAMASHITA et al.¹⁵⁾ Hydrolyzed solution was mixed with an equal volume of 20% trichloroacetic acid, held for 2 hours at room temperature and filtered. Degree of hydrolysis is based on the ratio of nitrogen amount in 10% TCA soluble fraction vs. that in whole hydrolyzate.

Hydration capacity was determined as described by BRYANT et al.¹⁶⁾ 0.2 g of dried plastein product was hydrated with 15 ml of solution of various pH, from pH 2 to 12, for 2 hours, then centrifuged at $2400 \times G$ for 30 min and the supernatant was carefully

removed with a pipette. Hydration capacity was calculated subtracting to the initial 15 ml, the volume found in the supernatant.

Plastein productivity was measured only as an index, according to the method reported by YAMASHITA et al.¹⁷⁾.

Recovery was calculated from the initial protein content in water soluble protein or waste protein and the protein content in dried plastein products. Result was presented as %.

Experimental Procedure.

Fresh sardine was obtained from Fish Market and freezed until use. Frozen sardine was defrosted in current water and immediatly processed. From sardine were obtained fillets and waste material such as head, visceras etc.

Water soluble protein extraction. Fillets were minced in automatic chopper, mixed with twice volume of water (w/v), homogenized with Ultra Turrax homogenizer for 3 min, held at 3°C with magnetic stirring for 1 hour, and centrifuged at 5000 rpm for 20 min. Supernatant was filtered with cotton used as a filter and this resultant solution was called Water Soluble Protein (WSP).

Waste material preparation. Whole sardine waste, head, visceras, and bones, was minced same as fillets and mixed with twice volume (w/v) of 0.24 N HCl¹⁶⁾ and homogenized with Ultra Turrax homogenizer. This preparation was used in the next step of hydrolysis.

Hydrolysis. The pH of water soluble protein was adjusted to 1.6 with HCl solution, treated with pepsin (Wako Pure Chemical Industries, digestive power 1: 10,000) 2/100 (enzyme/protein ratio) at 37°C, with stirring for 24 hours. Neutralization was made adjusting the pH to 7.0 with NaOH solution. Cooled in a water ice bath and held at this condition for one night, then was centrifuged. Supernatant solution was filtered using cotton as a filter and this resultant solution was called hydrolyzed solution.

Concentration. Hydrolyzed solution was concentrated at 40–45°C in a rotary evaporator, until protein concentration became 30–45%. The concentration and hydrolysis of waste material was carried out same as was described for water soluble protein.

Plastein reaction. Concentrated solutions from water soluble protein and waste material were used as substrates in plastein reaction. The substrates were incubated with Biopraxe (Nagase and Co. Ltd. 10,000 PUN/g) or Molsin (Seishin Pharmaceutical Co. Ltd.), proteases obtained from *Bacillus subtilis* and *Aspergillus saitoi* respectively, under the following conditions, concentration of protein 30–45%, enzyme substrate ratio, 2/100, pH 6.0³⁾, incubation temperature, 37°C and incubation time, 24 hours without stirring.

Preparation of ethanol-normal hexane insoluble products. Each incubation mixture was treated with twice volume of ethanol, stirred and centrifuged. Precipitate was treated with twice volume of normal hexane at 70°C for 10 min and centrifuged.

The precipitate was treated with ethanol, centrifuged and freeze dried. Obtained powder like product was used for analysis.

Results

Chemical composition. Table 1 shows lipid and protein composition of dried plastein products. Plastein products from water soluble protein showed higher protein content than plastein from waste material, 78.44–83.02% and 51.43–43.75% respectively.

Table 1. Protein and lipid content of plastein products (%).

	Biopraxe WSP**	plastein waste	Molsin WSP**	plastein waste
Dried matter	87.5	90.22	89.97	93.2
Protein*	78.44	51.43	83.02	43.75
Lipid	0.36	0.5	0.39	0.59

* Nitrogen \times 6.25

** Water soluble protein

All the samples showed a negligible amount of lipids between 0.3–0.5%, although lipid content of plastein from water soluble protein was less than plastein from waste material 0.36–0.39% and 0.5–0.59% respectively.

Lipids could be removed from water soluble protein by hydrolysis and plastein reaction only in 57.5%, for that reason was necessary to treat plastein products with normal hexane (Table 2).

Table 2. Lipids removal from fresh fish by plastein reaction, compared with n-hexane treatment.

Material	Lipid content (%)	Efficiency*** (%)
Fresh fish	12.0	—
Sample-1*	6.9	42.5
Sample-2**	0.36	97.0

* Dried plastein products without n-hexane treatment.

** Dried plastein products with n-hexane treatment.

*** Percent of lipids removal

Thiobarbituric acid number (TBA N), was low 9.39–7.25 for biopraxe plastein and molsin plastein from water soluble protein respectively. After 3 months of storage either in plastein products from water soluble protein or plastein products from waste protein, only a slight change in TBA N was detected. The rate of oxidation during plastein preparation was very high (Table 3).

These results suggest that the rate of oxidation of lipids during plastein preparation

Table 3. Thiobarbituric number (TBA N) of dried plastein products and fresh material.

Material	TBA number*	TBA number after 3 months
Water soluble protein		
Biopraxe plastein	9.39	10.38
Molsin plastein	7.25	8.37
Fresh material	4.25	—
Waste material	8.14	10.04
Molsin plastein	8.8	8.51

* mg of malonaldehyde/1000 g of sample.

Table 4. Protein digestibility* of plastein products.

Plastein products	Protein digestibility (%)
Biopraxe plastein WSP**	73.62
Molsin plastein WSP**	73.02
Biopraxe plastein-waste	70.18
Molsin plastein-waste	70.73
Casein (MERK)	88.28

* In vitro, using the multienzyme technique.⁸⁾

** Water soluble protein.

is faster than during preservation. Table 4 shows in vitro digestibilities of plastein products using trypsin, chymotrypsin and peptidase¹¹⁾. Digestibility of plastein products prepared from water soluble protein were a little higher than those prepared from waste material, 73.62–73.02% and 70.18–70.73% respectively. All the samples examined showed a protein digestibility lower than standard Merk casein. YAMASHITA et al.¹⁹⁾ reported similar digestibility value for plastein product obtained from soy bean. It is possible to suggest the same as YAMASHITA et al.¹⁹⁾ did with soy plastein product, that water soluble protein plastein and waste plastein are applicable to food.

Amino acids content. Amino acids composition of plastein products from water soluble protein and waste material, prepared using biopraxe and molsin was determined and presented in Table 5. The level of amino acids in plastein products made from water soluble protein and waste material of oily fishes approaches nearly to FAO/WHO (1973) pattern²⁰⁾.

The limiting essential amino acids in WSP-plastein compared with FAO/WHO (1973) pattern²⁰⁾, were leucine, phenylalanine and tryptophan. In waste protein was tryptophan.

Molsin plastein products from water soluble protein and waste protein, were limiting in tyrosine. Molsin plastein product from WSP was limiting also in isoleucine.

Molsin plastein products presented more limiting essential amino acids than biopraxe

Table 5. Amino Acid Composition of Plastein products, FAO/WHO Provisional Pattern (1973) (grs. aa/100 grs. protein).²⁰⁾

Amino Acid	Water Soluble Protein		Wastes Protein		— FAO/WHO Pattern
	Biopraxe Plastein	Molsin Plastein	Biopraxe Plastein	Molsin Plastein	
Essential aa					
Isoleucine	4.85	3.60	5.80	4.12	4.0
Leucine	5.80	4.20	7.73	7.96	7.0
Lysine	8.81	7.60	7.94	7.72	5.4
Total aromatic aa	7.93	5.51	7.41	6.68	6.1
Phenylalanine	4.63	2.75	4.36	5.16	3.05
Tyrosine	3.30	2.76	3.05	1.52	3.05
Total sulfur aa	4.52	5.16	4.27	4.46	3.5
Cystine	2.12	3.42	1.67	1.67	1.69
Methionine	2.40	1.74	2.60	2.77	
Threonine	4.05	3.92	5.10	5.07	4.0
Tryptophan	—	—	—	—	1.0
Valine	6.09	8.40	5.04	6.76	5.0
Nonessential aa					
Arginine	4.54	3.94	5.63	5.47	5.2
Glycine	5.77	5.63	7.26	6.70	2.2
Aspartic acid	10.88	11.02	9.98	9.13	7.7
Serine	3.85	4.57	4.39	4.30	7.7
Histidine	9.69	8.93	3.88	4.23	2.5
Alanine	5.80	5.68	3.98	5.67	6.1
Glutamic acid	12.42	13.19	14.24	15.85	14.7
Proline	3.01	6.31	5.85	6.65	10.7
Ammonia	1.98	2.29	1.46	1.54	

plastein products independently of utilized material (WSP or waste material).

In view to study some nutritonal properties of plastein products, based on amino acids composition, Table 6 shows E: N (ratio of essential amino acids to non essential amino acids), E: T (ratio of essential amino acids to total amino acids), E: P (ratio of essential amino acids to protein, 100 g), EAAI (essential amino acid index) is based on the ratios of the amounts of essential amino acids in a protein, relative to their amount in whole egg protein²¹⁾. These results were analyzed as described by SIKKA et al.²³⁾. SIKKA et al.²³⁾ have suggested that E: N, E: T, E: P, and EAAI have some relation with the high PER (protein efficiency ratio) and NPR (net protein retention) found in FPC (fish protein concentrate) prepared from picked ribbonfish and catfish. Plastein products prepared with molsin, either using water soluble protein or waste material, showed lower E: N, E: P, E: T and EAAI than plastein products prepared with biopraxe. Some relation exist between the amino acids

Table 6. E: N, E: P, E: T, ratios and EAAI (essential amino acid index) of plastein products.

Material	E: N	E: P	E: T	EAAI (%)
Bioprased plastein				
WSP**	0.75	0.42	0.421	82.29
Waste	0.764	0.43	0.433	84.7
Molsin plastein				
WSP**	0.624	0.38	0.384	75.13
Waste	0.718	0.42	0.418	83.69

* Nomenclature in the text

** See table 4

content of plastein products and the enzyme utilized in plastein reaction. YAMASHITA^{24,25)} and ONOUE¹⁹⁾ reported that hydrolyzed solutions and plastein products showed some differences in amino acids content, specially in hydrophobic and hydrophilic amino acids. Some properties of plastein products, such as solubility is influenced by the enzyme utilized in the process²⁶⁾, and now the result suggest that the enzyme utilized in plastein reaction have also influence in nutritional aspects such as amino acids content and E: N, E: P, E: T, EAAI ratios in plastein products.

Hydration capacity. Plastein products didn't show a high hydration capacity value, compared with FPC prepared by low temperature alcohol treatment, which value was about 30 at pH 2.0²⁷⁾. Bioprased plastein from water soluble protein showed the highest values at pH extremes 2 and 12. At those same pH bioprased plastein from waste material showed the lowest value. Results suggest that hydration capacity is more dependent of the protein material utilized in plastein reaction than the enzyme utilized in plastein reaction (Fig. 1).

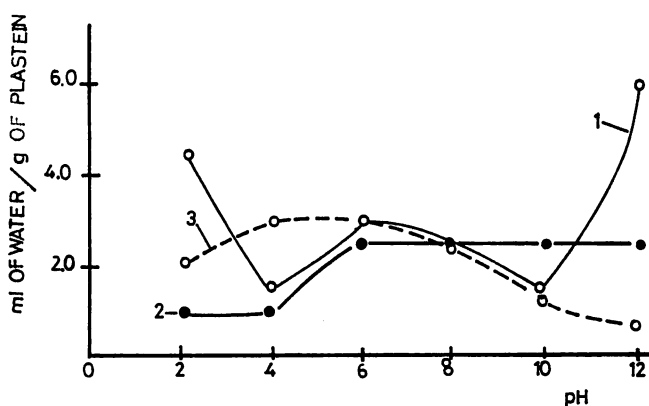
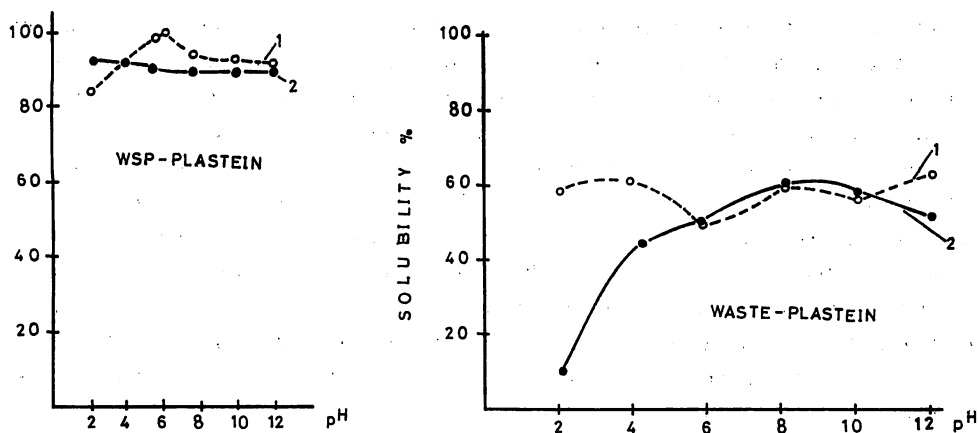


Fig. 1. Hydration capacity of dried plastein products at various pH.

1. Bioprased plastein-water soluble protein.
2. Molsin plastein-waste
3. Bioprased plastein-waste

Although plastein products didn't present a high hydration capacity value, was possible to get gel like products. Solubility profile of plastein products. Plastein products are defined as water insoluble products^{28,29}. EDWARDS et al²⁶ reported that the solubility profile of plastein products at various pH, was dependent on the enzyme utilized in plastein reaction. YAMASHITA et al.²³, prepared a glutamic acid enriched plastein with great solubility, and these plastein products showed a low molecular weight compared with standard plastein, being this one of the reason for the great solubility.

Results show that solubility profile of plastein products prepared with molsin or biopraxe from water soluble protein and waste material was dependent on the enzyme utilized in plastein reaction and on the molecular weight distribution of hydrolyzate. Fig. 2 and Fig. 3 show that plastein products prepared using molsin in plastein reaction were more soluble than those prepared using biopraxe.



Figs. 2, 3. Solubility profile of dried plastein products.

1. Molsin plastein product.
2. Biopraxe plastein product.

Fig. 2. Plastein from water soluble protein.

Fig. 3. Plastein from waste material.

Solubility profile of low molecular weight distribution plastein products (molecular weight distribution from tyrosine 182 to bacitracin 1422) and high molecular weight distribution plastein (molecular weight distribution from tyrosine 182 to lysozyme 14.700) was different. At pH 8 and 4, high molecular plastein showed a markedly insolubility compared with low molecular weight plastein. Results are in agreement with the two above mentioned authors^{23,25} (Fig. 4).

Molecular weight distribution of hydrolyzed solution and plastein products, prepared from water soluble protein and waste material of sardine.

Fig. 5, 6, 7 and 8, show molecular weight distribution of hydrolyzed solution and plastein products prepared from water soluble protein of sardine. In Fig. 5 and 6,

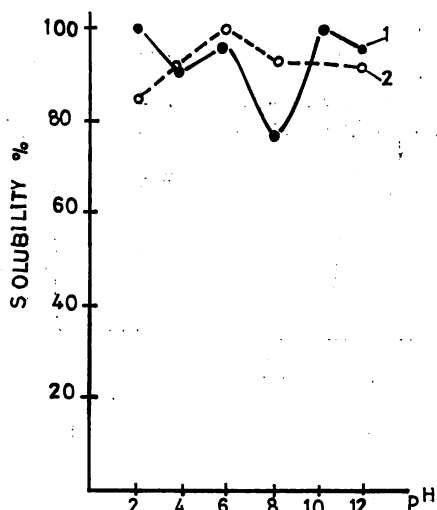


Fig. 4. Solubility profile of bioprased plastein products.

1. Plastein product with high molecular weight distribution (from tyrosine 182 to lysozyme 14,700)
2. Plastein product with low molecular weight distribution (from tyrosine 182 to bacitracin 1,422)

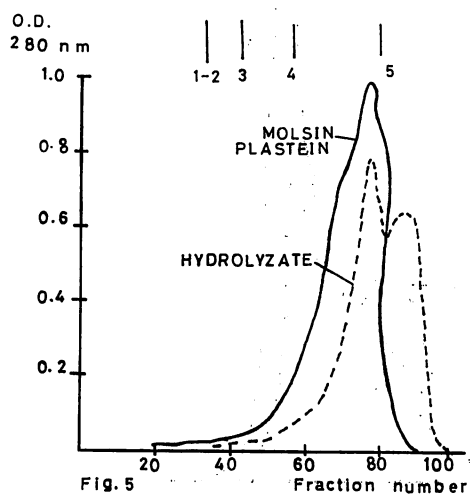


Fig. 5

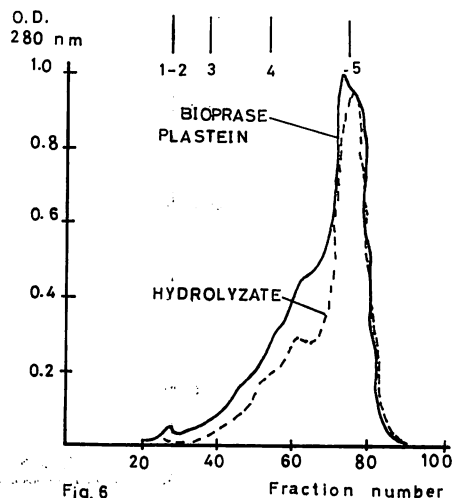


Fig. 6

Figs. 5, 6. Chromatography of hydrolyzates and plastein products on sephadex G-50. (Water soluble protein)

Degree of hydrolysis: 80%

Eluent solution: 50% acetic acid, elution rate: 0.33 ml/min, column size: (2.8 × 98) cm.

Fig. 5. 30% of substrate concentration and molsin in plastein reaction.

Fig. 6. 45% of substrate concentration and bioprased in plastein reaction.

The numbers denote elution positions of standard compound: 1. chymotrypsinogen (MW 25,000), 2. lysozyme (MW 14,700), 3. insulin (MW 5,730), 4. bacitracin (MW 1,422), 5. tyrosine (MW 182).

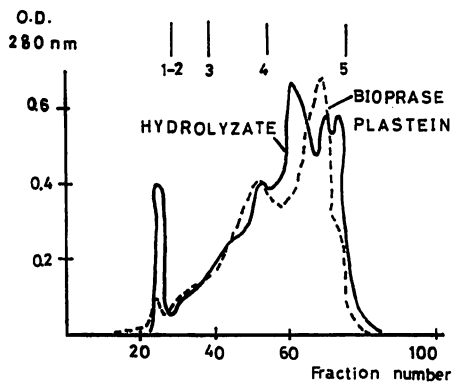


Fig. 7

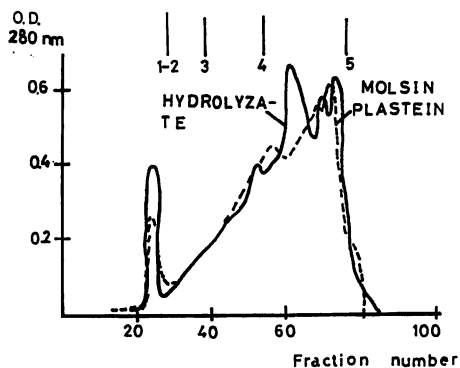


Fig. 8

Figs. 7, 8. Chromatography of hydrolyzates and plastein products on sephadex G-50. (Water soluble protein)

Degree of hydrolysis: 46%

Fig. 7. 40% of substrate concentration and bioprase in plastein reaction.

Fig. 8. 40% of substrate concentration and molsin in plastein reaction

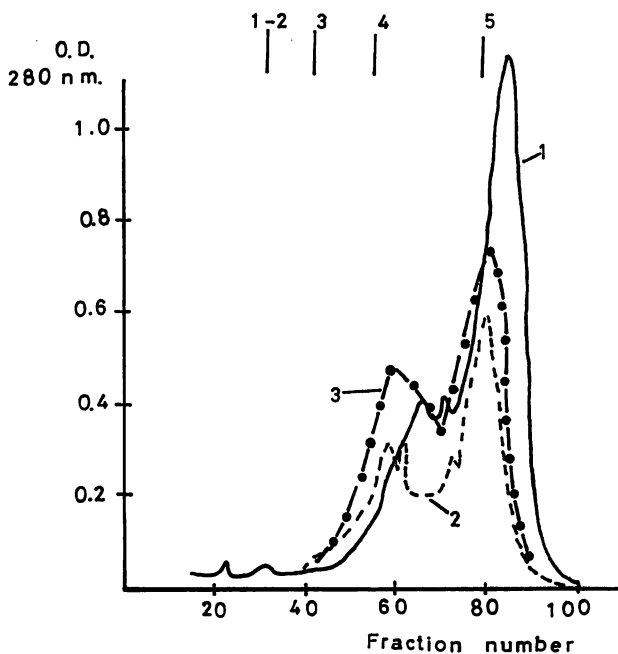


Fig. 9. Chromatography of hydrolyzate and plastein products on sephadex G-50. (Waste material)

Degree of hydrolysis: 95%

Substrate concentration during plastein reaction: 40%

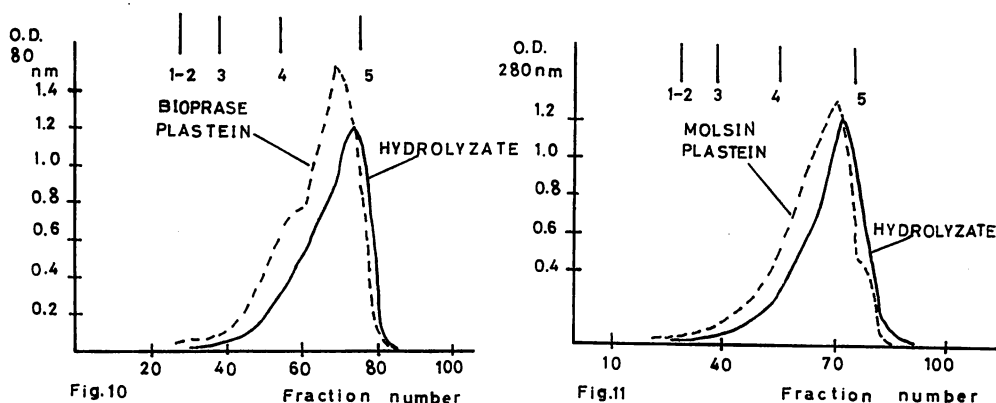
1. Hydrolyzate.

2. Bioprase plastein product.

3. Molsin plastein product.

the degree of hydrolysis was 80%, and in Fig. 7 and 8 the degree of hydrolysis became 46%. Results showed that in both conditions of hydrolysis (46% and 80%), and using either biopraser or molsin in plastein reaction, only a slight change in molecular weight distribution was detected between hydrolyzed solutions and plastein products.

Fig. 9, 10, and 11, show molecular weight distribution of hydrolyzed solutions and plastein products, prepared from waste material of sardine. In Fig. 9, the degree of hydrolysis was 95%, and in Fig. 10, 11, the degree of hydrolysis was 79%. The results were similar to those reported for water soluble protein. No appreciable change was detected in molecular weight distribution between hydrolyzed solution and plastein product.



Figs. 10, 11. Chromatography of hydrolyzates and plastein products on sephadex G-50. (Waste material)

Degree of hydrolysis: 79%

Fig. 10. 40% of substrate concentration and biopraser in plastein reaction.

Fig. 11. 40% of substrate concentration and molsin in plastein reaction.

These results are in agreement with those reported by HOFTEEN *et al.*¹⁾, EDWARDS *et al.*³⁰⁾ and MONTI³¹⁾, which reported that no high molecular weight material was formed by plastein reaction. Influence of substrate concentration and degree of hydrolysis on gel formation during plastein reaction.

1) In water soluble protein.

Using 30% of substrate concentration, with degree of hydrolysis of 80% and molsin in plastein reaction, condition which was reported by TSAI *et al.*³⁾, as the most favourable for plastein reaction, although was plastein formation, showed by the increase in O.D. at 600 nm, YAMASHITA *et al.*³²⁾, was not possible to get gel like products.

Increasing the concentration of substrate to 45% with the same degree of hydrolysis 80%, either using molsin or biopraser in plastein reaction was possible to get gel like products (Table 7).

Table 7. Influence of substrate concentration and degree of hydrolysis on gel formation during plastein reaction.

1) Water soluble proetin				
Enzyme	Substrate concentration (%)	Degree of hydrolysis (%)	Molecular weight distribution	Gel formation
Molsin	30	80	from 182 to 1,422	negative
Molsin	45	80	from 182 to 1,422	positive
Molsin	40	46	from 250 to 14,700	positive
Biopraser	40	46	from 250 to 14,700	positive
Control	40	46	from 250 to 14,700	negative
2) Waste material				
Molsin	45	95	from 150 to 1,000	negative
Molsin	45	95	from 150 to 1,000	negative
Molsin	40	79	from 200 to 1,422	positive
Biopraser	40	79	from 200 to 1,422	positive
Control	40	79	from 200 to 1,422	negative

2) In waste material.

Using 45% of substrate concentration, with degree of hydrolysis of 95%, neither with molsin or biopraser was possible to get gel like products.

Using 40% of substrate concentration, with degree of hydrolysis of 79%, either with molsin or biopraser was possible to get gel like products.

These results confirm the importance of substrate concentration and molecular weight distribution on gel formation. Similar results were reported by EDWARDS et al.²⁶⁾ in egg albumin, and TSAI et al.³⁾ in soy bean protein (Table 7).

Recovery.

Protein from water soluble protein of sardine (*Sardinops melanosticta*) could be recovered in 61.5% and 67.8%, using molsin and biopraser respectively. These values were similar to protein recovery from fish meat by ethanol and n-hexane at low temperature treatment²⁷⁾.

Protein from waste material could be recovered only in 28.23% and 22.6%, using molsin and biopraser respectively. These values were near to those reported by ONOUE¹⁹⁾ using *Petrale sole*, which recovery was 35% (Table 8).

Table 8. Protein recovery in sardine using plastein reaction.

Enzyme and material utilized in plastein reaction	Recovery (%)
Water soluble protein	
Molsin	61.5
Biopraser	67.8
Waste material	
Molsin	28.23
Biopraser	22.6

Discussion

The low contents of lipids and the low increase in TBA number suggest, that is possible the storage of dried plastein products, prepared from water soluble protein and waste material of oily fish (sardine, *Sardinops melanosticta*).

The results in amino acids content and protein digestibility, the light white color and no fish odor found in plastein products, suggest that is possible to use these dried plastein products in food formulation. Although is necessary to study the technological compatibility of plastein products in food systems. More investigation is needed, specially related to gel formation and gel stability. In this report was not possible to get gel like products from dried plastein, only a caramel like product with high viscosity could be obtained by rehydration with water. Hoften et al.¹⁾ reported that, freeze dried plastein didn't lose its ability to form gel, when dissolved in the original volume of water, for that reason is possible that, ethanol and n-hexane treatment, affect the gel capacity recuperation of dried plastein products. No high molecular weight material was formed during plastein reaction of waste protein and water soluble protein of oily fish (sardine). This result is similar to those reported by others authors, using different substrates^{1,26,29}.

Results showed that solubility profile of plastein products were dependent of molecular weight distribution of hydrolyzed solutions and the enzyme utilized in plastein reaction. These results also were reported by YAMASHITA et al.²⁴⁾ in soy bean, and EDWARDS et al.²⁶⁾, in egg albumin.

Although plastein products were deficient in tryptophan, this amino acid, and other amino acids pattern can be incorporated same as other authors have already reported^{25,28,29}.

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