行政院國家科學委員會專題研究計畫　成果報告

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行政院國家科學委員會補助專題研究計畫 ☑ 成 果 報 告 □期中進度報告

鰻魚蛋白質酵素水解物抗氧化成分及其分離純化之探討

Separation and purification of antioxidative compounds in enzymatic hydrolyzates of eel protein

計畫類別：☑ 個別型計畫　□ 整合型計畫
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摘　　要

爲開發水產品作為天然抗氧化劑，本研究計畫擬以日本白鰻（*Anguilla japonica*）及其廢棄物之蛋白質抽出物為原料，利用 Papain、Protease N、Protease A 和 Prozyme 6 等酵素加以水解，探討水解物的抗氧化活性。鰻魚肉及其骨頭等廢棄物水解液之 pH 值於水解期間呈現先降後升之趨勢。氨含量則隨水解時間延長而增加，Papain 及 Protease N 酵素水解物在水解 6 小時內，可溶性蛋白質含量隨水解時間增長而增加，爾後降低。

鰻魚肉及其骨頭廢棄物在未水解前之游離胺基酸以牛磺酸、甘胺酸、組胺酸和丙胺酸為主，經酵素水解後，其游離胺基酸、肌肽、甲肌肽及其他胜肽類的含量皆隨水解時間的增加而增加，且高於未水解者。骨頭水解物之游離胺基酸含量高於魚肉水解物；而肌肽在魚肉水解物中的含量比骨頭來得高。甲肌肽被發現存於酵素水解液中，而在原本的魚肉及骨頭中皆未發現。酵素水解物在水解 6 小時內，胜肽含量隨水解時間增長而增加，爾後降低。胜肽類之複合胺基酸在魚肉水解物中遠較骨頭水解物為高。

不同檢測方法包括抑制亞麻油酸自氧化、捕捉 DPPH 自由基及螯合鐵離子等被使用來評估鰻魚水解物之抗氧化性，結果顯示酵素水解物具有較強的抗氧化能力，且骨頭水解物之抗氧化能力較魚肉水解物為佳。統計分析顯示酵素水解物抗氧化能力之強弱與胜肽類的含量有關。

利用膜及膠體過濾層析法劃分水解物分子量大小，分析比較其不同區分物與抗氧化活性之間的關係，分子量約在 5000 kDa 以下之胜肽類具有較強的抗氧化性。結果顯示分子量約為 1300 和 200 kDa 皆具有抗氧化性。本計畫結果有助於了解水解物具抗氧化性之有效成分及其作用機制，可作為未來開發萃取水產品蛋白質水解物製成具有抗氧化性與食療效果之天然添加物商品之參考。

關鍵詞：鰻魚、蛋白質水解物、抗氧化活性、游離胺基酸、胜肽類

ABSTRACT

The main objective of this research project is to develop the natural antioxidant from aquatic products. The hydrolysis of Japanese eel (*Anguilla japonica)* protein and their wastes protein is carried out by using Papain, Protease N, Protease A, and Prozyme 6. The antioxidative activities of the hydrolyzates are investigated. The pH value of hydrolysates decreased and then increased during hydrolysis. The NH_3 contents of the hydrolysates increased with the hydrolysis time; however, the soluble protein of Papain and Protease N hydrolysates increased at the 6 h hydrolysis and thereafter decreased.

The predominant FAAs of pre-hydrolyzed meat and bone were taurine, glycine, histidine, and alanine. Changes in the levels and compositions of FAAs, anserine, carnosine and other peptides during hydrolysis showed that their increased levels of the hydrolysates obtained with commercial enzymes were much higher than pre-hydrolyzed. Bone hydrolysates had more FAAs than meat hydrolysates. The meat contained the highest amount of carnosine than that bone. Anserine was not detectable in meat and bone, but they were found in considerable levels in enzyme hydrolysates. The peptides of the enzyme hydrolysates increased dramatically in the first 6 h and thereafter decreased with the increase of FAAs. The total peptides of the meat hydrolysate was slightly higher than those in the bone hydrolysate.

Different antioxidant measurements including the inhibition of linoleic acid autoxidation,

scavenging effect on α,α-diphenyl-β-picrylhydrazyl free radical, and chelating ability Fe^{2+} showed that eel hydrolysates possessed noticeable antioxidant activities. The bone hydrolysates possessed a little higher antioxidant activity than that of the meat hydrolysate. A good correlation existed between the amount of peptides in the hydrolysates and antioxidant activity.

The molecular size of protein hydrolyzate is determined by membrane and gel permeation chromatography, and the antioxidative activity of different fractions is also measured. The fraction with molecular weight <5000 kDa presented the highest antioxidant activity. Results revealed that the peptide with molecular weight of approximately 1,300 kDa possessed a stronger *in vitro* antioxidant activity than that of 200 kDa. This study will also provide the essential background and database for the efficient utilization of the hydrolysis protein extracts to manufacture the natural antioxidant.
Key words: Eel; Protein hydrolysate; Antioxidative activity; Free amino acids; peptides

INTRODUCTION

Oxidation is essential to living organisms to fuel biological processes. The uncontrolled production of free radical derived from oxidation may be associated with the onset of many diseases such as cancer, rheumatoid arthritis, and arteriosclerosis (Halliwell and Gutteridage, 1984). Almost organisms are well protected against free radical damage by enzymes such as superoxide dismutase and catalase, or antioxidant compounds such as ascorbic acid, tocopherols and glutathione (Aruoma, 1998). Therefore, the antioxidants present in human diet are of great interest as possible protective agents to help human body reduce oxidative damage. Addition of synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and tertiary butylhydroquinone can increase the shelf life of food products by improving the stability of lipids. However, these synthetic compounds were suspected to be carcinogen (Bran, 1975; Whysner et al., 1994). Therefore, the exploration of natural antioxidants has been of importance.

Various physiological activities of the proteolytic hydrolysates from food proteins have been detected. The antioxidative activity of hydrolysates such as soy protein (Chen et al., 1995), caseins and whey protein (Chiang and Chang, 2005), sunflower protein (Villanueva et al., 1999), egg-yolk protein (Park et al., 2001), bovine albumin (Hatate et al., 1990), pork protein (Carlsen et al., 2003), skin gelatin (Mendis et al., 2005), fish protein (Je et al., 2005; Wu et al., 2003) and chicken essence (Wu et al., 2005) have been reported. It is possible that the levels and compositions of FAAs and peptides may contain some information regarding antioxidant activities of protein hydrolysates. Thus, the antioxidant activities either of amino acids or peptides have been investigated to gain insight into the antioxidant mechanism of protein hydrolysates.

The present study reports on the antioxidant activities of eel meat and their waste hydrolysates prepared accelerated hydrolysis with the addition of commercially available protease. Changes in the levels and compositions of FAAs and small peptides during hydrolysis are also investigated to find out their relationships with antioxidant activities. The potent antioxidant peptide fractions are also isolated from the eel by size exclusion chromatography in the present study.

MATERIALS AND METHODS

1. Materials

Fresh Japanese eel were purchased from seafood markets in Keelung, Taiwan. The fishes, still in the rigor mortis stage at the time of purchase, were stored on ice during transportation to the laboratory. On arrival at the laboratory, the fishes were immediately decapitated and filleted, and the meat were collected and homogenized for enzymatic hydrolysis.

2. Preparation of eel protein hydrolysates

Eel meat and bone (250 g) were mixed with 500 g of distilled water and homogenized for about 2 min. Commercial enzymes (1.25 g) were added to the mixture for hydrolysis process at 50 ^{o}C for 0, 3, 6, 9, and 12 hrs. The conditions of enzymatic hydrolysis were adapted as the manufacturer recommended. At the end of hydrolysis period, the mixtures were heated in boiling water for 10 min to inactivate protease. The hydrolysates were centrifuged (10 min at 7,000 × g) and the supernatants were lyophilized and stored in a desiccator until use.

3. Extraction of free amino acids and peptides

Extracts of FAAs and peptides were prepared according to the method of Konosu et al. (1974).

4. Size exclusion chromatography

Peptide fractions of the hydrolysates were separated using column chromatography as described by Chen et al. (1995).

5. Determination of soluble protein

The soluble protein was determined according to the method of Lowry et al. (1951).

6. Free amino acids, carnosine and anserine

Analytical conditions and procedure were performed according to the report published by Shiau et al. (1996).

7. Amino acids of low-molecular-weight peptides

The difference between the value of amino acids by HCl hydrolysis and free amino acids were referred to as the constituent amino acids of low-molecular-weight peptides (Konosu and Yamaguchi, 1982; Chiou and Konosu, 1988).

8. Inhibition of linoleic acid autoxidation

The antioxidant activity of eel hydrolysates were determined according to the ferric thiocyanate method (Chen et al., 1995).

9. Scavenging effect on DPPH free radical

The scavenging effect of the hydrolysates on DPPH free radical was measured by Shimada et al.(1992.

10. Chelating of metal ions

The chelating of Fe^{2+} by the sample was estimated by the method of Dinis et al. (1994).

RESULTS AND DISCUSSION

1. Changes in soluble protein contents during hydrolysis

The soluble protein contents of eel meat during hydrolysis are shown in Fig. 1. The

concentration of soluble protein was about 5~18 mg/g before hydrolysis, and the amount was increased after hydrolysis. It increased dramatically in the first 6 h, and gradually decreased thereafter. Soluble proteins of hydrolysates with papain and Protease N were found in higher levels than other hydrolysates during the first 6 h hydrolysis. Although Protease A and Prozyme 6 both showed soluble protein during hydrolysis, their contents were much lower as compared to those of papain and Protease N.

2. Changes in free amino acids, anserine and carnosine during hydrolysis

Changes in FAA constituents in the meat and bone eel during hydrolysis by employing Protease N are shown in Table 1 and 2. The total FAAs increased dramatically in the first 6 h, and then decreased during the elongated hydrolysis. Most of the individual FAAs increased during hydrolysis, and leucine increased particularly much more than other FAAs. Leucine and phenylalanine increased much more than other FAAs, which accounted for 25~27% of the total FAAs in the meat and bone. As compared the changes of FAAs with papain and Protease N, both enzyme hydrolysates had the similar trend during hydrolysis. In general, the FAAs in the hydrolysate of bone were higher than that of meat.

The dipeptides, anserine, was not found in the muscle of eel. However, they were found in considerable levels in the meat and bone hydrolysates of papain, Protease N, Protease A, and Prozyme 6. Although the production mechanisms of anserine and carnosine are not clear, the antioxidant properties of the dipeptides have been studied extensively. Boldyrev et al. (1988) demonstrated that anserine and carnosine could decrease membrane lipid/oxidation rates by measuring thiobarbituric acid reactive substance. Chan et al. (1994) indicated that carnosine-related dipeptides possessed the antioxidant abilities under the phosphatidylcholine liposome model, and discovered that the formation of the •OH free radicals could be detained by carnosine.

3. Changes in low-molecular-weight peptides during hydrolysis

Changes in levels of peptides in the meat and bone during hydrolysis by employing Protease N are shown in Table 3 and 4, respectively. The peptides increased dramatically in the first 6 h, and gradually decreased thereafter. Glutamic acid, aspartic acid, lysine, and glycine were the major constituted amino acids of small peptides in the Protease N hydrolysates. The total amount of peptides in the meat and bone hydrolysates after 5 h were 11079 mg/100 mL and 2606 mg/100 mL, accounting for 92 and 16 times higher than that in pre-hydrolyzed sample. The change of constituted amino acids of peptides in the Protease N hydrolysates was similar to that in the other hydrolysates.

4. Antioxidant activity of eel hydrolysates

The inhibition of linoleic acid autoxidation of hydrolysates by papain, Protease N, Protease A, and Prozyme 6 increased dramatically in the first 6 h, and gradually decreased thereafter (Fig. 2). This trend was similar to the changes of peptides over the duration. As compared with 0 h, both eel hydrolysates from meat and bone accelerated process had higher inhibition. This result illustrated that eel hydrolysate with protease possessed a noticeable antioxidant property to inhibit the peroxidation of linoleic acid.

Fig. 3 shows the hydrolysates derived from protease hydrolysis were capable of quenching

DPPH radical, and the scavenging ability of bone hydrolysis was higher than the meat. The trends show that the scavenging effect for all hydrolysates increased slightly in the first 3 h, and remained unchanged during the elongated period of hydrolysis. In general, the scavenging DPPH radical of the bone hydrolysate was higher than that of the meat hydrolysate. The result revealed that the eel hydrolysates might contain electron-donating substances, which could react with free radicals, convert them to more stable products and terminate radical chain reaction. As shown in Table 4, there existed good correlations between the amounts of combined amino acids and histidine-related dipeptides in eel hydrolysates and their scavenging the DPPH radical. Thus, a higher peptide level of the hydrolysate possessed a stronger antioxidant activity. Erickson and Hultin (1992) indicated that, under inhibitory conditions, histidine-related compounds capable of inhibiting lipid peroxidation might stem from its ability to coordinate with iron, thus preventing reduction of Fe^{3+} to Fe^{2+}. The fact that the enzymatic hydrolysates possessed DPPH free radical quenching activity and reducing power may be associated with carnosine and anserine.

Fig. 4 shows the chelating effect of papain, Protease N, Protease A, and Prozyme 6 hydrolysates on ferrous ions. The meat and bone eel hydrolysates had similar chelating effect on ferrous ion. The Prozyme 6 hydrolysate possessed the greatest chelating ability of metal ions among all the hydrolysates. Metal ions are essential needs for health maintenance because human body requires them for oxygen transport, respiration, and the activity of many enzymes. However, metal ions are extremely reactive and will catalyze oxidative changes in lipids, proteins, and other cellular components.

5.Distribution of molecular weight of peptide fractions

According to the previous studies, the 6 h hydrolysates of eel bone showed the highest antioxidant activities, and the low-molecular-weight peptides were present in high levels in the enzyme hydrolysates. In order to explore more insight into the antioxidant mechanism, the 6 h hydrolysates were chosen as samples to analyze their molecular weight distributions of low-molecular-weight peptides. The peptide fractions of the bone eel hydrolysates separated by size exclusion chromatography on Sephadex G-25 are shown in Fig. 5. The fraction with molecular weight <5000 kDa presented the highest antioxidant activity (Fig. 6). Four and/or five peak peptide fractions were observed in the hydrolysates with papain, Protease N, Protease A and Prozyme 6. Results revealed that the peptide with molecular weight of approximately 1,300 kDa possessed a stronger *in vitro* antioxidant activity than that of 200 kDa.

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計畫成果自評

本研究計畫以日本白鰻之肌肉及骨頭廢棄物之蛋白質抽出物為原料，利用 Papain、Protease N、Protease A 和 Prozyme 6 等酵素加以水解，發現骨頭廢棄物蛋白質之低分子量胜肽類含量甚豐，其抗氧化性與胜肽類含量多寡有良好的正相關性，但與總游離胺基酸含量相關性小，具強抗氧化性的部分可能與含有較多量之肌肽、甲肌肽及小分子胜肽類有關。其結果可作為開發水產物蛋白質水解物作為天然抗氧化性劑之水解方法，並且有效利用養殖漁獲物以提昇其附加價值，可作為未來開發萃取水產品蛋白質水解物製成抗氧化劑兼具調味功能之天然添加物商品之參考。本研究成果內容適合於學術期刊中發表，以了解其抗氧化作用機制與特性，建立魚肉水解物生產具有抗氧化性與醫療效果之胜肽類天然添加物商品之資料。

附表(圖)

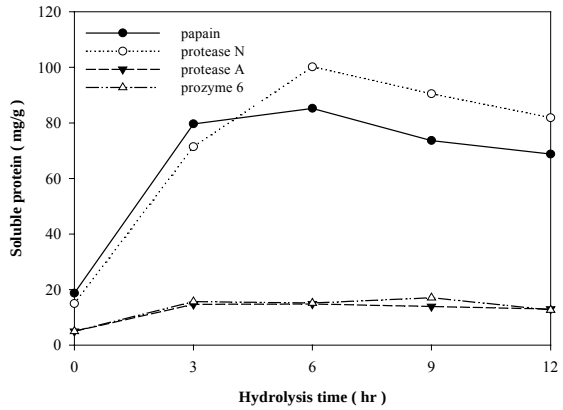

Fig. 1. Changes in soluble protein contents of eel meat hydrolysates during hydrolysis.

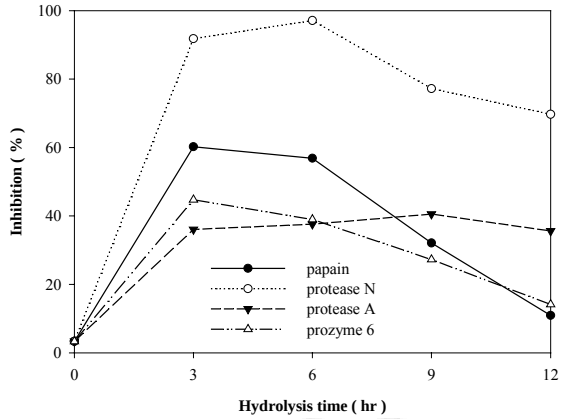

Fig. 2. Effect of hydrolysis method and time on the autoxidation of linoleic acid of eel bone hydrolysates.

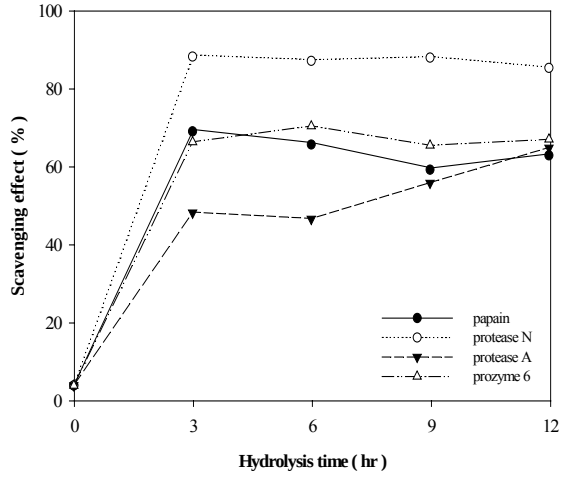

Fig. 3. Influence of hydrolysis method and time of eel bone hydrolysates on scavenging effect of DPPH radical.

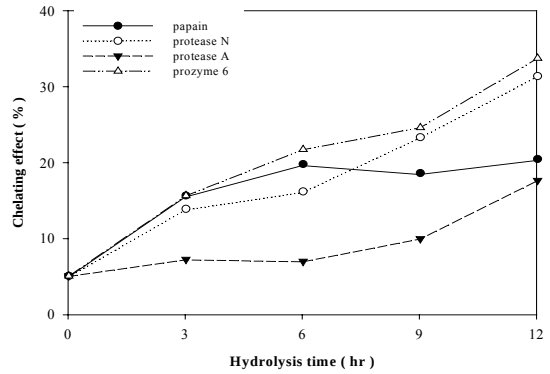

Fig. 4. Effect of hydrolysis method and time of eel bone hydrolysates on chelating of ferrous ion.

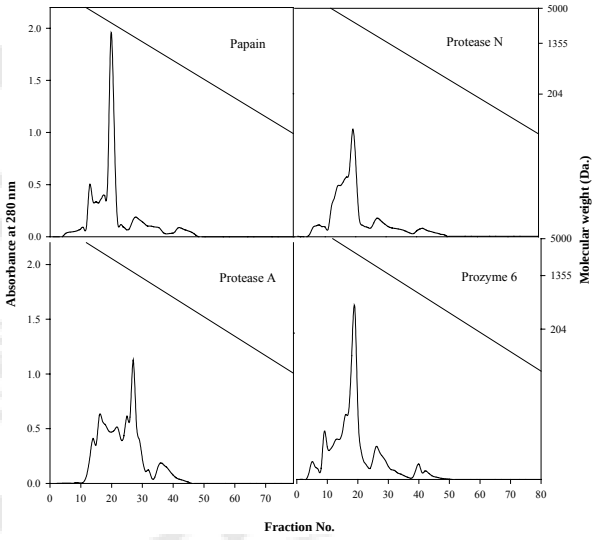

Fig. 5. The bone eel hydrolysates on Sephadex G-25.

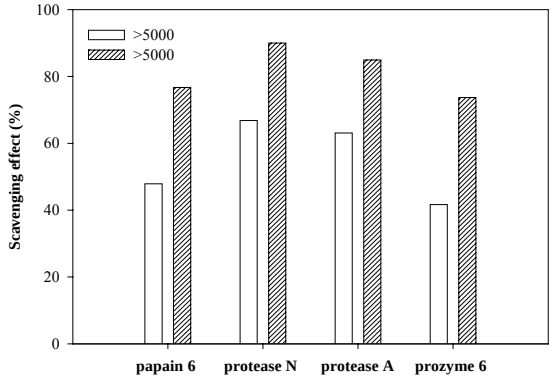

Fig. 6. Influence of hydrolysis method of eel bone hydrolysates on scavenging effect of (DPPH) radical.

Table 1. Changes in free amino acids and related compounds in eel meat hydrolysates during hydrolysis with Protease N

(mg/100 ml)

	Hydrolysis time (hr)				
	0	3	6	9	12
Taurine	46.19	7.05	18.05	11.71	8.34
Aspartic acid	0.72	25.76	18.52	9.70	5.86
Threonine	3.31	49.22	21.33	14.58	7.84
Serine	2.03	41.07	22.65	13.68	8.54
Glutamic acid	3.63	50.39	23.21	13.27	8.01
Proline	3.85	11.53	7.42	3.71	2.75
Glycine	15.56	20.90	14.43	8.68	6.26
Alanine	11.09	65.59	55.73	31.83	22.55
Valine	5.53	67.21	55.59	30.47	22.15
Methionine	—	56.86	62.68	33.44	28.42
Isoleucine	2.80	74.08	67.15	37.50	30.03
Leucine	3.48	163.31	179.35	103.33	79.55
Tyrosine	1.50	59.68	51.61	25.15	23.80
Phenylalanine	1.58	82.08	98.02	54.18	46.24
β-Alanine	7.84	13.11	17.43	2.52	8.41
Lysine	7.44	109.61	49.37	30.40	21.82
Histidine	10.11	41.57	35.80	23.33	17.92
Arginine	1.83	100.89	46.48	27.42	19.98
Total	142.31	1129.84	1017.36	577.79	446.60
Anserine	—	38.49	43.77	32.43	25.58
Carnosine	472.70	95.98	148.41	107.48	89.52

Table 2. Changes in free amino acids and related compounds in eel bone hydrolysates during hydrolysis with Protease N

(mg/100 ml)

	Hydrolysis time (hr)				
	0	3	6	9	12
Taurine	5.72	19.98	41.58	16.54	11.65
Aspartic acid	0.12	5.77	29.99	20.09	10.77
Threonine	0.43	17.83	51.70	40.97	24.36
Serine	0.32	16.83	51.68	40.01	23.51
Glutamic acid	0.82	10.55	34.51	23.01	17.59
Proline	0.27	4.52	10.91	7.19	4.56
Glycine	1.01	10.93	32.54	26.07	15.51
Alanine	1.20	41.43	124.33	90.83	55.67
Valine	0.43	38.84	128.30	88.87	55.44
Methionine	0.02	38.42	109.01	64.41	41.71
Isoleucine	0.28	42.09	121.94	85.34	54.41
Leucine	0.42	107.85	292.78	173.35	117.18
Tyrosine	0.20	34.83	96.28	59.19	37.79
Phenylalanine	0.19	60.99	157.81	82.04	56.69
β-Alanine	1.93	19.17	44.98	22.05	15.44
Lysine	0.55	37.30	101.37	73.12	45.85
Histidine	2.33	37.68	104.23	67.63	44.41
Arginine	0.29	40.35	113.72	78.94	45.97
Total	16.80	684.29	1800.46	1149.15	745.76
Anserine	—	26.86	63.72	27.91	17.56
Carnosine	24.89	73.14	140.21	56.20	42.96

Table 3. Changes in constituted amino acids and related compounds in eel meat hydrolysates during hydrolysis with Protease N

(mg/100 ml)

	Hydrolysis time (hr)				
	0	3	6	9	12
Taurine	—[2]	43.42	37.99	33.37	31.52
Aspartic acid	1.81	1204.74	1142.85	866.77	863.77
Threonine	0.89	481.57	453.42	350.24	340.49
Serine	0.86	447.02	383.79	323.39	313.52
Glutamic acid	4.53	1955.62	1806.43	1441.75	1392.78
Proline	2.53	—	448.30	402.70	404.91
Glycine	13.49	769.83	763.37	561.16	558.66
Alanine	3.27	735.43	735.57	552.21	532.21
Valine	0.66	555.80	572.04	408.75	395.98
Methionine	0.51	265.46	268.10	220.47	189.89
Isoleucine	0.62	553.16	505.39	403.75	385.31
Leucine	1.34	857.05	840.22	655.40	600.08
Tyrosine	0.05	268.95	319.07	243.27	209.60
Phenylalanine	0.65	419.03	435.43	326.77	294.37
β-Alanine	26.77	129.70	120.35	112.51	91.03
Lysine	1.55	993.49	978.69	727.49	731.48
Histidine	58.73	536.50	492.81	403.47	401.35
Arginine	1.75	770.26	726.12	580.37	554.62
Total	120.93	11078.76	11158.58	8725.82	8385.08

Table 4. Changes in constituted amino acids and related compounds in eel bone hydrolysates during hydrolysis with Protease N (mg/100 ml)

	Hydrolysis time (hr)				
	0	3	6	9	12
Taurine	5.43	—	6.68	—	—
Aspartic acid	4.96	311.74	468.75	358.14	296.27
Threonine	2.18	108.93	169.79	105.92	86.55
Serine	2.75	111.16	169.98	96.48	83.81
Glutamic acid	10.38	507.13	749.39	596.50	476.82
Proline	8.00	195.03	299.90	230.69	186.66
Glycine	29.86	311.02	469.12	357.29	297.24
Alanine	9.13	200.53	306.12	180.66	153.37
Valine	2.12	110.70	189.04	98.45	80.32
Cystine	0.32	0.61	40.21	—	35.39
Methionine	0.74	29.93	115.98	23.55	54.08
Isoleucine	1.32	87.04	164.85	83.98	70.29
Leucine	2.55	109.01	229.17	37.31	66.05
Tyrosine	0.43	40.44	89.86	—	26.40
Phenylalanine	1.54	48.34	111.71	8.87	29.15
β-Alanine	28.83	7.08	27.68	13.19	2.90
Lysine	4.06	220.97	333.77	227.10	175.41
Histidine	41.99	93.92	152.07	70.18	57.45
Arginine	5.51	189.00	297.96	163.96	141.92
Total	165.98	2605.70	4336.30	2593.52	2254.73