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Production of Free Amino Acids and γ-Aminobutyric Acid by Autolysis Reactions from Wheat Bran

YOICHI NOGATA* AND TAKASHI NAGAMINE

National Agricultural Research Center for Western Region, 6-12-1 Nishifukatsu, Fukuyama, Hiroshima 721-8514, Japan

To find added value for wheat-milling byproduct, an approach for producing free amino acids and γ-aminobutyric acid (GABA) was examined. Milled whole grain, bran, shorts, red dog, and 60% extracted flour all released amino acids using a water-soaking treatment. Little difference was found in amino acid production yield from whole grain between the soft and hard wheat cultivars investigated. Among the milled fractions, shorts produced the largest amount of total amino acids followed by bran, red dog, and 60% extracted flour in decreasing order. From the byproduct fraction (mixture of bran and shorts), leucine (Leu), arginine (Arg), valine (Val), lysine (Lys), glutamine (Gln), phenylalanine (Phe), isoleucine (Ile), and GABA were produced at 486, 421, 316, 329, 321, 279, 227, and 118 mg/100 g, respectively, in 120 h at 40 °C. Optimal pH for the byproduct fraction was 3.5−5.0 for α-amino acids and 5.5 for GABA. The production levels rose with increasing temperature up to 40−50 °C for α-amino acids and up to 40 °C for GABA. The yield of all amino acids increased in the experimented period until 120 h except for aspartic acid (Asp) and asparagine (Asn). Thus, wheat-milling byproducts have the potential to become effective materials for developing foods enriched in branched-chain amino acids (BCAA), Arg, Lys, Gln, Phe, and GABA.

KEYWORDS: Bran; shorts; *Triticum aestivum*; autolysis; amino acids; γ-aminobutyric acid; byproduct

INTRODUCTION

The outer parts of the starchy endosperm of wheat seed, called bran and shorts, are recognized as byproducts in the wheat-milling industry because adulteration with these byproducts negatively affects the color of flour and the physiological properties of processed products. These fractions account for about 30% of seed weight, and their yearly production in Japan is about 1.4 million tons. Because most of these byproducts are usually used as feed for livestock, their effective utilization is of great interest for the wheat-milling industry. One possible approach is to add value through finding biological activities associated with these byproducts.

Wheat-milling byproducts are known to contain not only soluble and insoluble fiber but also other biologically active components such as phytic acid (*1*), ferulic acid derivatives (*2*), lutein (*3*), anthocyanins (*4*), proanthocyanidins (*5*), and tocotrienols (*6*). Some approaches have been conducted to fortify such compounds in seeds. The contents of γ-aminobutyric acid (GABA) and phytic acid were increased in germinated wheat by manipulating the dissolved oxygen level (*7*). Saikusa et al. reported that GABA was released into water from milled rice germ (*8*). The beneficial effects of amino acids have been investigated extensively. GABA has been reported to have a reductive effect on blood pressure (*9*), which has been attributed to a vascular dilatation effect (*10*), a sympatholytic effect (*11*), and a suppressive effect on antidiuretic hormone secretion (*12*) by GABA. With regard to proteinous amino acids, leucine (Leu) (*13*), glutamine (Gln) (*14*), and arginine (Arg) (*15*) have been reported to promote protein synthesis. Arg, a conditionally essential amino acid, has also been shown to stimulate the secretion of growth hormone and insulin (*16*) and to improve immunological function (*17*). Furthermore, amino acid supplements including branched-chain amino acids (BCAA), Arg, and Gln showed a quicker recovery from muscle fatigue following eccentric exercise training (*18*).

Mature wheat seeds contain proteolytic enzymes such as serine, aspartic, and metallo- and thiol-proteases (*19, 20*) in parts of the aleurone, testa, and embryo. In addition, wheat bran has been reported to contain carboxypeptidase (*21*) and aspartic proteinase (*22*). On the other hand, about one-third of the endospermal reserve proteins are localized in the living cells of the aleurone layer in barley grains (*23*). Thus, wheat-milling byproducts have the potential to produce amino acids by autolysis reaction. The application of autolysis reaction has advantages such as its being a simple process and inexpensive costs, which would lead to an effective utilization of milling byproducts.

In this study, we have evaluated the production of proteinous amino acids and GABA from milled seed parts using whole

* Corresponding author (e-mail ynogata@affrc.go.jp; telephone +81 84 923-4100; fax +81 84 924-7893).

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Table 1. Change of Free Amino Acid Concentrations Produced from Milled Whole Meal after 1 h of Reaction

	soft wheat							hard wheat						
	N17	Fuku	Mina	Shira	K107	Chiku	av	C152	C140	S180	Haru	Shikan	Roblin	av
aliphatic amino acids														
Gly	3.7 ± 0.3[a]	3.4 ± 0.3	4.8 ± 0.5	3.5 ± 0.3	3.7 ± 0.4	4.2 ± 0.4	3.9 ± 0.5	3.2 ± 0.3	3.5 ± 0.3	3.2 ± 0.4	3.7 ± 0.4	2.7 ± 0.2	3.6 ± 0.3	3.3 ± 0.4
Ala	3.6 ± 0.4	3.7 ± 0.4	6.2 ± 0.6	3.8 ± 0.3	4.3 ± 0.5	4.1 ± 0.5	4.3 ± 1.0	4.2 ± 0.2	4.5 ± 0.3	4.6 ± 0.3	5.9 ± 0.5	3.2 ± 0.2	4.0 ± 0.3	4.4 ± 0.9
Val	3.0 ± 0.1	2.2 ± 0.2	5.0 ± 0.4	2.3 ± 0.2	4.4 ± 0.3	4.4 ± 0.4	3.6 ± 1.2	2.2 ± 0.1	3.5 ± 0.2	3.6 ± 0.2	4.0 ± 0.2	3.0 ± 0.1	2.8 ± 0.1	3.2 ± 0.7
Ile	2.5 ± 0.3	1.3 ± 0.2	2.8 ± 0.2	2.4 ± 0.2	2.8 ± 0.2	2.7 ± 0.2	2.4 ± 0.6	2.6 ± 0.3	2.6 ± 0.3	2.2 ± 0.3	2.6 ± 0.3	2.0 ± 0.2	2.1 ± 0.1	2.4 ± 0.3
Leu	5.1 ± 0.2	5.4 ± 0.2	7.3 ± 0.5	5.3 ± 0.2	5.8 ± 0.4	6.1 ± 0.5	5.8 ± 0.8	5.6 ± 0.2	6.0 ± 0.2	5.4 ± 0.3	5.5 ± 0.2	3.9 ± 0.1	4.0 ± 0.1	5.1 ± 0.9
dicarboxylic amino acids and amides														
Asp	0.4 ± 0.1	0.5 ± 0.3	−1.0 ± 0.3	−1.3 ± 0.1	−1.5 ± 0.6	−1.1 ± 0.2	−0.7 ± 0.9	−1.8 ± 0.1	−0.8 ± 0.2	0.0 ± 0.1	1.9 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.0 ± 1.3
Glu	6.6 ± 0.7	4.5 ± 0.5	4.9 ± 1.0	6.9 ± 0.8	4.2 ± 0.5	2.2 ± 0.5	4.9 ± 1.7	6.1 ± 0.9	6.1 ± 0.2	4.0 ± 0.4	2.3 ± 0.8	3.7 ± 0.4	8.7 ± 0.7	5.2 ± 2.3
Asn	2.5 ± 0.3	1.0 ± 0.3	4.2 ± 0.9	2.1 ± 0.4	2.6 ± 0.7	5.1 ± 0.9	2.9 ± 1.5	1.2 ± 0.9	1.7 ± 0.4	1.6 ± 0.3	4.1 ± 0.5	2.2 ± 0.2	1.7 ± 0.2	2.1 ± 1.0
Gln	2.2 ± 0.4	3.6 ± 0.1	4.8 ± 0.9	2.2 ± 0.4	3.7 ± 0.2	6.6 ± 0.3	3.9 ± 1.7	3.3 ± 0.6	3.3 ± 1.1	2.4 ± 0.1	5.1 ± 0.9	1.8 ± 0.6	4.0 ± 0.2	3.3 ± 1.2
basic amino acids														
Arg	7.2 ± 0.7	4.8 ± 0.8	11.7 ± 1.3	5.1 ± 0.6	9.7 ± 1.1	9.7 ± 1.1	8.0 ± 2.8	7.2 ± 0.8	9.0 ± 0.4	7.9 ± 1.0	9.5 ± 1.2	6.1 ± 0.4	8.7 ± 0.7	8.1 ± 1.3
Lys	6.4 ± 0.5	4.0 ± 0.4	6.6 ± 0.7	4.4 ± 0.5	4.8 ± 0.6	7.2 ± 0.6	5.6 ± 1.3	3.3 ± 0.4	6.9 ± 0.5	4.7 ± 0.6	5.3 ± 0.5	5.0 ± 0.5	2.9 ± 0.2	4.7 ± 1.5
His	2.8 ± 0.4	1.8 ± 0.2	2.1 ± 0.3	1.7 ± 0.2	2.1 ± 0.2	2.5 ± 0.3	2.2 ± 0.4	0.6 ± 0.1	2.6 ± 0.2	1.4 ± 0.2	1.9 ± 0.3	1.4 ± 0.2	1.5 ± 0.1	1.6 ± 0.7
aromatic amino acids														
Trp	4.5 ± 0.3	4.5 ± 0.3	4.8 ± 0.1	5.2 ± 0.2	6.0 ± 0.4	4.2 ± 0.1	4.9 ± 0.7	5.2 ± 0.4	2.9 ± 0.2	4.1 ± 0.5	5.8 ± 0.3	5.2 ± 0.5	5.5 ± 0.3	4.8 ± 1.1
Phe	3.8 ± 0.2	3.5 ± 0.1	5.4 ± 0.6	3.6 ± 0.2	4.2 ± 0.6	3.8 ± 0.5	4.1 ± 0.7	4.0 ± 0.1	4.1 ± 0.2	3.8 ± 0.3	3.6 ± 0.4	2.1 ± 0.1	1.6 ± 0.1	3.2 ± 1.1
Tyr	2.7 ± 0.2	2.7 ± 0.1	4.2 ± 0.3	4.0 ± 0.2	2.7 ± 0.4	3.3 ± 0.4	3.3 ± 0.7	3.5 ± 0.1	3.6 ± 0.2	4.3 ± 0.4	3.1 ± 0.5	0.6 ± 0.0	0.7 ± 0.0	2.6 ± 1.6
sulfur, hydroxyl, and cyclic amino acids														
Met	1.7 ± 0.1	1.9 ± 0.1	2.7 ± 0.3	2.0 ± 0.1	2.4 ± 0.2	2.0 ± 0.5	2.1 ± 0.4	2.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.2	2.2 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.9 ± 0.3
Ser	2.8 ± 0.2	2.8 ± 0.3	4.2 ± 0.4	2.6 ± 0.2	3.1 ± 0.3	3.8 ± 0.4	3.2 ± 0.6	2.8 ± 0.2	3.1 ± 0.2	2.5 ± 0.2	3.6 ± 0.3	2.3 ± 0.2	2.8 ± 0.2	2.9 ± 0.5
Thr	2.0 ± 0.2	1.9 ± 0.2	2.4 ± 0.2	1.7 ± 0.1	1.6 ± 0.2	2.0 ± 0.2	1.9 ± 0.3	1.9 ± 0.1	2.1 ± 0.2	1.7 ± 0.2	1.9 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.8 ± 0.2
Pro	6.4 ± 0.3	6.1 ± 0.4	7.3 ± 0.5	5.3 ± 0.5	5.5 ± 0.5	6.7 ± 0.7	6.2 ± 0.8	5.3 ± 0.3	5.8 ± 0.3	4.0 ± 0.4	6.6 ± 0.9	4.0 ± 0.1	6.0 ± 0.5	5.3 ± 1.1
γ-amino acid														
GABA	7.7 ± 0.6	5.8 ± 0.5	12.9 ± 1.3	5.0 ± 0.4	6.2 ± 0.7	8.6 ± 1.1	7.7 ± 2.9	6.4 ± 0.6	6.3 ± 0.6	5.3 ± 0.5	8.9 ± 0.8	5.7 ± 0.5	4.9 ± 0.6	6.3 ± 1.4
α-amino acid total	69.9 ± 7.0	59.6 ± 5.8	90.4 ± 10.6	62.8 ± 6.8	72.1 ± 9.1	79.5 ± 9.3	72.4 ± 11.3	62.4 ± 7.4	72.6 ± 6.2	63.4 ± 7.9	78.6 ± 9.1	52.6 ± 5.2	64.2 ± 7.0	65.6 ± 9.0

[a] Mean of four replicates (mg/100 g).

grain, bran, shorts, red dog, and 60% extracted flour by autolysis reactions and investigated the optimization of the reaction conditions.

MATERIALS AND METHODS

Seed Samples and Preparation of Milled Fractions. Soft wheats Norin 17 (N17), Fukusayaka (Fuku), Minaminokaori (Mina), Shirasagikomugi (Shira), Kanto 107 (K107), and Chikugoizumi (Chiku) and hard wheats Chugoku 152 (C152), Chugoku 140 (C140), Saikai 140 (S140), Haruyutaka (Haru), Shikan, and Roblin were grown and harvested at the field of the National Agricultural Research Center for Western Region in Fukuyama, Hiroshima, in 2006 and 2007. Each cultivar was grown by two replications and put together for experiment. Grains of Fuku were milled in a Bühler test mill (Bühler Inc., Switzerland), and 20% bran, 10% shorts, 10% red dog, and 60% extracted flour were obtained from the outer parts of the seeds. The milling process was conducted at 20 °C and 60% humidity using grain tempered to 14.5% water content. The bran fraction consisted of peel and part of the aleurone layer, shorts from the aleurone layer and germ, red dog mainly of the endosperm and part of the aleurone layer, and 60% extracted flour from the endosperm. Whole grain samples were prepared for each experiment using a centrifugal mill (model ZM 200, Retsch, Germany) using a 0.5 mm screen at room temperature. All samples were confirmed not to be damaged by preharvest sprouting by checking the falling number (*24*) and the initial concentration of GABA. Milled samples were kept at −30 °C until use.

Reaction and Quantification of Amino Acids. Portions (0.2 g) of the milled fractions were suspended in 4.0 mL of 50 mM sodium phosphate (pH 5.5) and reacted in a water bath with a wrist action shaker at 40 °C. After reaction, 4.0 mL of 16% trichloroacetic acid was added, the sample was centrifuged at 10000*g* for 20 min, and the supernatant was filtered through a 0.5 μm membrane filter followed by amino acid analysis. The quantification of amino acids was performed using a model L-8800 amino acid auto analyzer (Hitachi, Japan). Authentic amino acid standards were purchased from Wako Pure Chemicals (Tokyo, Japan). For each sample, the amount of released amino acid was calculated by subtracting the blank value. Pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), phenylmethanesulfonyl fluoride (PMSF), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (St. Louis, MO). Pepstatin A, E-64, PMSF, and EDTA were utilized at final concentrations of 1.46, 20, 1000, and 1000 μM, respectively. Each inhibitor was added to the reaction bufferm and the reaction was performed in the same manner as described above.

RESULTS AND DISCUSSION

Production of Amino Acids from Milled Whole Grain. To clarify the differences in amino acid producing ability among wheat cultivars, changes in amino acid concentrations were examined in suspensions of milled whole grain after autolysis reaction for 1 h (**Table 1**). Soft wheat samples tended to produce larger amounts of total amino acids than hard wheat samples on average, although the differences were not significant. Mina produced the largest amount of total α-amino acids and GABA, and the difference in total α-amino acids was about 38 mg/100 g compared to the amount produced by Shikan, which produced the smallest amount. The concentrations of all amino acids increased in both soft and hard wheat cultivars except for aspartic acid (Asp), which showed constant or slightly decreased levels compared to blank value. The production of large amounts of aliphatic amino acids, basic amino acids, methionine (Met), serine (Ser), threonine (Thr), proline (Pro), and GABA was common to cultivars producing a large amount of total α-amino acid such as Mina, K107, Chiku, C140, and Haru. Among amino acids, Leu, Arg, Pro, and GABA were produced at greater than 5 mg/100 g on average in cultivars investigated. Although the reason for the difference in amino acid producing ability is not clear, the ratio of embryo in a seed as well as the concentration of proteinase in it would affect the result (*20*).

Differences in the Amino Acid Releasing Ability of Milled Fractions. Differences in the amino acid producing activities of the milled parts of Fuku seeds are shown in **Table 2**. In the amino acid producing activity, a substantial difference was observed between the byproduct fractions (bran and shorts) and inner fractions (red dog and 60% extracted flour). The shorts

Table 2. Differences in the Amino Acid Producing Activity of Milled Fractions from Fuku after 1 h of Reaction

	bran	shorts	red dog	60% extracted flour
aliphatic amino acids				
Gly	4.6 ± 0.3[a]	9.4 ± 0.4	0.6 ± 0.1	0.1 ± 0.0
Ala	5.3 ± 0.2	6.1 ± 0.2	1.2 ± 0.1	0.6 ± 0.1
Val	6.4 ± 0.1	10.3 ± 0.3	1.3 ± 0.1	0.3 ± 0.0
Ile	4.2 ± 0.2	6.8 ± 0.2	0.9 ± 0.1	0.0 ± 0.0
Leu	10.7 ± 0.4	15.6 ± 0.5	2.8 ± 0.2	0.6 ± 0.1
dicarboxylic amino acids and amides				
Asp	−5.2 ± 0.4	−1.5 ± 0.2	0.3 ± 0.0	0.1 ± 0.0
Glu	−10.9 ± 0.8	−23.9 ± 1.9	0.9 ± 0.2	0.4 ± 0.0
Asn	12.3 ± 0.6	12.1 ± 0.5	1.3 ± 0.3	0.3 ± 0.1
Gln	12.7 ± 0.5	8.0 ± 0.4	0.1 ± 0.0	0.4 ± 0.1
basic amino acids				
Arg	10.9 ± 0.2	15.5 ± 0.3	2.1 ± 0.1	2.1 ± 0.1
Lys	7.4 ± 0.1	15.6 ± 0.3	0.1 ± 0.0	0.0 ± 0.0
His	1.8 ± 0.0	3.8 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
aromatic amino acids				
Trp	6.2 ± 0.5	5.1 ± 0.4	1.9 ± 0.3	0.0 ± 0.0
Phe	6.6 ± 0.2	9.9 ± 0.4	2.0 ± 0.1	0.5 ± 0.0
Tyr	3.0 ± 0.2	6.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
sulfur, hydroxyl, and cyclic amino acids				
Met	3.4 ± 0.2	5.3 ± 0.2	0.5 ± 0.1	0.4 ± 0.1
Ser	4.9 ± 0.2	7.3 ± 0.3	0.6 ± 0.1	0.4 ± 0.0
Thr	3.4 ± 0.2	5.9 ± 0.3	0.7 ± 0.1	0.0 ± 0.0
Pro	7.4 ± 0.3	4.8 ± 0.4	1.9 ± 0.2	0.4 ± 0.0
γ-amino acid				
GABA	12.6 ± 0.4	51.5 ± 2.2	5.5 ± 0.3	1.3 ± 0.1
α-amino acid total	95.1 ± 5.4	123 ± 8.9	19.2 ± 1.6	6.6 ± 0.5

[a] Mean of four replicates (mg/100 g).

Table 3. Optimum pH for the Production of Amino Acids from the Byproduct Fraction of Fuku

	pH							
	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5
aliphatic amino acids								
Gly	84[a]	95	97	100	92	88	—[b]	—
Ala	42	77	100	95	86	41	—	—
Val	21	59	100	96	73	37	—	—
Ile	28	67	87	100	96	61	—	—
Leu	53	81	96	100	91	44	—	—
dicarboxylic amino acids and amides								
Asp	91	98	100	96	90	81	—	—
Glu	92	97	100	86	64	43	—	—
Asn	40	56	81	100	86	62	—	—
Gln	91	97	100	93	89	86	—	—
basic amino acids								
Arg	42	93	100	94	44	—	—	—
Lys	51	82	100	92	41	—	—	—
His	40	62	92	100	94	81	66	—
aromatic amino acids								
Trp	—	—	54	65	100	97	77	59
Phe	58	81	100	87	74	38	—	—
Tyr	62	91	100	91	78	60	—	—
sulfur, hydroxyl, and cyclic amino acids								
Met	43	66	92	100	94	56	—	—
Ser	60	100	96	81	42	—	—	—
Thr	56	97	100	95	83	47	—	—
Pro	41	69	100	95	83	47	—	—
γ-amino acid								
GABA	—	—	19	51	89	100	95	47

[a] Mean relative values (%) of three replicates. [b] Not measured.

fraction produced the largest amount of each amino acid except for dicarboxylic amino acids and amides, tryptophan (Trp), and Pro. More than 10.0 mg/100 g of valine (Val), Leu, asparagine (Asn), Arg, and lysine (Lys) and more than 50.0 mg/100 g of GABA were produced from this fraction with a 1 h reaction. Such a short time frame increase in amino acid concentration suggests that proteolytic and decarboxylic enzymes in the shorts acted on coexisting proteins. The composition of the α-amino acids produced from the bran fraction was similar to that of shorts. This fraction produced Leu, Asn, Gln, Arg, and GABA at more than 10.0 mg/100 g. As with the milled whole grain sample, the concentration of Asp did not clearly increase in each individual fraction. The decrease of glutamic acid (Glu) in the bran and shorts fractions was considered to be due to its consumption by glutamate decarboxylase (EC 4.1.1.15.) to produce GABA. The red dog fraction produced GABA at 5.5 mg/100 g, although the production levels of other amino acids were less than 3.0 mg/100 g. The 60% extracted flour fraction produced only 8.1 mg/ 100 g of amino acids in total. As the whole grain of Fuku produced a relatively small amount of amino acids (**Table 1**), milled fractions of other cultivars such as Mina, Chiku, and Haru would produce larger amounts of amino acids. The relation of amino acid producing ability and wheat kernel characteristics, namely, weight, size, testing weight, protein content, and so on, will not be clear until further study is conducted. From these results, bran and shorts are considered to be effective sources for amino acid production.

Optimum pH and Temperature for the Production of Amino Acids. Relative values for the production of amino acids from the byproduct fraction at different pH values at 40 °C in a 4 h reaction are shown in **Table 3**. Unlike other amino acids, GABA was produced in its highest amounts at pH 5.5−6.0, which agreed with a report evaluating the accumulation of GABA in rice germ (*8*). All other amino acids had pH optima at 4.0−4.5 except for Trp and Ser. Morris et al. reported that endopeptidases prepared from wheat embryos had pH optima at 4.0 (*20*). On the other hand, the concentration of soluble proteins was reported to be increased by acidification of the aleurone layer (*25*). Weakly acidic conditions may be effective for not only enzyme activation but also the provision of substrates. Amino acids, such as Ala, Val, Leu, Glu, Arg, Lys, Phe, Ser, Thr, and Pro, were produced at rates of less than 50% of their maximal amount at pH 5.5. Conversely, GABA was produced at a rate of about 50% at pH 4.5. When the reaction was performed at pH 5.0, all amino acids except Glu, Arg, Lys, and Ser were produced at rates of more than 70% of their maximal amounts. From these results, reaction at pH 5.0 was considered to be suitable for the production of both α-amino acids and GABA. The optimum temperature for the production of amino acids at pH 5.5 for 4 h was between 40 and 50 °C except for Glu (**Table 4**). As Glu was converted to GABA by glutamic acid decarboxylase at the early stage of reaction, its concentration was lowest at 40 °C, which was the optimum temperature for the production of GABA. At 45 °C all amino acids were produced at more than 75% of their maximum yield except for Glu.

Effect of Class-Specific Inhibitors on the Production of Amino Acids. The effect of potential inhibitors of the production of amino acids from the bran fraction of Fuku in a 4 h reaction is summarized in **Table 5**. Because the concentrations of both Asp and Glu decreased during reaction (**Table 2**), their inhibition rates were not examined. The production of all α-amino acids except for Trp and Pro was substantially inhibited by either inhibitor. As expected, the production of GABA was not affected by any of the inhibitors. PMSF, which is a specific inhibitor of serine proteases, decreased the production of each α-amino acid except for Trp, although its effect was not as strong as those of pepstatin A or EDTA. Umetsu et al. reported that at least four carboxypeptidases exist in wheat bran (*21*). The activity of the main peptidase was inhibited by diisopropylfluorophosphate, which is a specific inhibitor of serine proteases. Although it is difficult to identify the enzymes from this result, those inhibited

Table 4. Optimum Temperature for the Production of Amino Acids from the Byproduct Fraction of Fuku

	temperature						
	30 °C	35 °C	40 °C	45 °C	50 °C	55 °C	60 °C
aliphatic amino acids							
Gly	68[a]	81	97	100	92	67	30
Ala	58	65	91	100	85	63	30
Val	33	61	74	96	100	86	52
Ile	32	58	67	94	100	81	43
Leu	30	52	63	91	100	90	57
dicarboxylic amino acids and amides							
Asp	92	98	100	94	92	91	91
Glu	73	71	64	67	76	96	100
Asn	61	82	100	76	50	36	21
Gln	50	77	92	100	94	84	75
basic amino acids							
Arg	56	81	97	100	92	64	31
Lys	70	90	100	83	71	52	32
His	21	31	48	100	94	59	20
aromatic amino acids							
Trp	83	94	100	91	72	64	56
Phe	22	37	56	92	100	86	68
Tyr	23	39	56	95	100	92	76
sulfur, hydroxyl, and cyclic amino acids							
Met	23	51	67	92	100	85	64
Ser	52	82	100	97	91	60	23
Thr	52	83	100	95	84	55	28
Pro	69	81	96	100	87	72	56
γ-amino acid							
GABA	60	80	100	94	86	63	33

[a] Mean relative values (%) of three replicates.

Table 5. Effect of Protease Inhibitors on the Production of Amino Acids from the Byproduct Fraction of Fuku

	inhibition rate (%)			
	PMSF	pepstatin A	EDTA	E-64
aliphatic amino acids				
Gly	32[a]	13	62	ni[b]
Ala	27	34	58	ni
Val	26	35	46	ni
Ile	23	39	51	ni
Leu	18	44	20	2.9
dicarboxylic amino acids and amides				
Asp	—[c]	—	—	—
Glu	—	—	—	—
Asn	4.0	13	80	ni
Gln	36	19	70	ni
basic amino acids				
Arg	19	30	17	3.6
Lys	28	31	18	2.5
His	39	31	26	ni
aromatic amino acids				
Trp	ni	5.0	ni	ni
Phe	18	45	17	ni
Tyr	23	41	26	ni
sulfur, hydroxyl, and cyclic amino acids				
Met	25	34	23	ni
Ser	29	31	56	0.5
Thr	32	31	51	ni
Pro	8.1	ni	ni	ni
γ-amino acid				
GABA	3.7	ni	3.4	ni

[a] Mean relative values of three replicates. [b] No inhibition. [c] Not measured.

by PMSF might be the same as the previously reported peptidases. Pepstatin A inhibited the production of α-amino acids by more than 30% except for glycine (Gly), dicarboxylic amino acids, Trp, and Pro. The presence of aspartic proteinase(s) and its (their) participation in hydrolyzing endogenous proteins is supported by the report of an aspartic proteinase from wheat bran hydrolyzing bran globulin and being inhibited by pepstatin A (*22*). Belozersky et al. also reported that wheat seed aspartic proteinase released fragments with molecular masses of 57−63 and 24.5−26.6 kDa from wheat gliadins, suggesting that this enzyme plays a role in the initial step of proteolysis of storage proteins in the early period of germination (*26*). Proteolytic enzymes might act against bran proteins in the same manner during the water-soaking reaction method used in this study. EDTA exhibited the highest inhibitory effect on aliphatic and dicarboxylic amino acids, Ser, and Thr except for Leu. The susceptibility of proteases to EDTA increased in the late stages of wheat seed development, showing the accumulation of metalloproteases in mature grains (*19*). Metalloproteases in wheat bran are considered to be responsible for the hydrolysis of bran protein as well as serine and aspartic proteases. E-64, an inhibitor of cysteine proteases, did not exhibit an inhibitory effect on the production of amino acids. A low level of cysteine proteinases exists in developing wheat grains (*19*), whereas most cysteine proteinases are synthesized de novo during seed germination (*27*−*29*). As a result, these proteases are unlikely to play a major role in protein hydrolysis in this study.

Table 6. Influence of the Addition of NaClO on the Production of Amino Acids in 120 h of Reaction from the Byproduct Fraction of Fuku

	[NaClO]			
	0 ppm	10 ppm	50 ppm	100 ppm
aliphatic amino acids				
Gly	163 ± 31[a]	183 ± 33	131 ± 29	75 ± 15
Ala	329 ± 29	349 ± 35	343 ± 35	156 ± 15
Val	313 ± 26	320 ± 29	297 ± 27	142 ± 13
Ile	225 ± 30	230 ± 28	206 ± 24	102 ± 14
Leu	475 ± 39	489 ± 34	481 ± 34	196 ± 18
dicarboxylic amino acids and amides				
Asp	81 ± 23	63 ± 16	90 ± 24	77 ± 22
Glu	343 ± 34	355 ± 39	361 ± 40	116 ± 13
Asn	78 ± 21	82 ± 23	81 ± 23	64 ± 18
Gln	302 ± 35	328 ± 43	308 ± 40	57 ± 7
basic amino acids				
Arg	462 ± 36	457 ± 32	473 ± 33	189 ± 17
Lys	312 ± 38	339 ± 44	327 ± 36	156 ± 20
His	171 ± 30	162 ± 26	155 ± 25	40 ± 7
aromatic amino acids				
Trp	67 ± 18	67 ± 19	53 ± 16	15 ± 4
Phe	288 ± 17	281 ± 14	292 ± 18	100 ± 7
Tyr	243 ± 23	238 ± 19	232 ± 24	52 ± 5
sulfur, hydroxyl, and cyclic amino acids				
Met	101 ± 15	96 ± 15	41 ± 7	6 ± 1
Ser	185 ± 26	171 ± 21	189 ± 25	72 ± 11
Thr	153 ± 35	147 ± 35	165 ± 36	58 ± 13
Pro	323 ± 34	309 ± 28	325 ± 29	61 ± 7
γ-amino acid				
GABA	148 ± 11	123 ± 10	29 ± 4	13 ± 1
α-amino acid total	4610 ± 645	4670 ± 677	4550 ± 694	1730 ± 260

[a] Mean of three replicates (mg/100 g).

Influence of the Addition of Sodium Hypochlorite (NaClO). Diluted NaClO solution is used as a germicidal agent in the food industry. To understand the influence of NaClO on the production levels of amino acids, different concentrations of NaClO treatment were examined. The addition of 10 and 50 ppm (available chloride) of NaClO produced about the same amount of total α-amino acid as the control, whereas the addition of 100 ppm of NaClO affected the production level with less than 40% of the control (**Table 6**). Among α-amino acids, the concentration of Met was obviously decreased in a NaClO concentration-dependent manner, which might be due to the oxidation of Met. The production level of GABA was also inhibited at 50 ppm and higher concentrations of NaClO, suggesting that glutamate decarboxylase was susceptible to

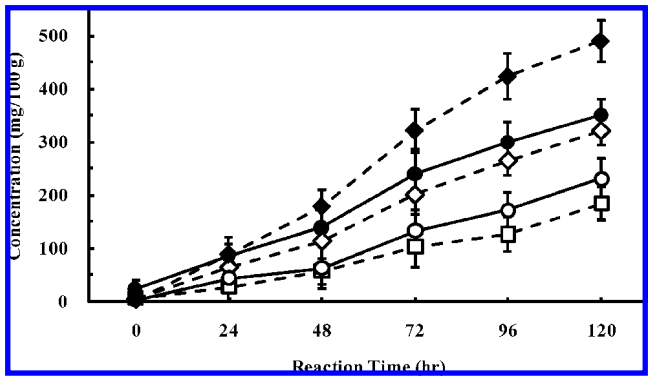

Figure 1. Time-dependent changes of aliphatic amino acid concentrations produced from the byproduct fraction of Fuku: □, Gly; ●, Ala; ◇, Val; ◆, Leu; ○, Ile.

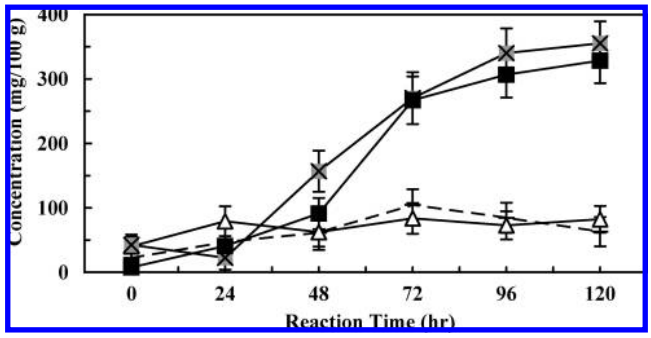

Figure 2. Time-dependent changes of dicarboxylic amino acid and amide concentrations produced from the byproduct fraction of Fuku: △, Asn; +, Asp; ×, Glu; ■, Gln.

inhibition by NaClO. From these results, the addition of 50 ppm of NaClO was considered to be suitable for the production of α-amino acids, whereas the production of GABA was limited.

Time-Dependent Production of Amino Acids from the Byproduct Fraction. The byproduct fraction was subjected to reaction in the presence of 10 ppm of NaClO, and amino acid concentrations were analyzed periodically up to 120 h. Aliphatic amino acids were one of the most abundantly produced groups, and the concentration of all amino acids consistently increased during the experimental period. The amounts of Leu, Ala, Val, Ile, and Gly produced were 475, 329, 313, 227, and 163 mg/100 g, respectively, at 120 h (**Figure 1**). The extended reaction time allowed for further increases in each amino acid. In the case of dicarboxylic amino acids and amides, the concentration of Glu decreased slightly in the first 24 h and then started increasing until the end of the reaction (**Figure 2**). The concentration of Gln, recognized as a conditionally essential amino acid (*30*), showed a slow increase until 48 h and rapidly increased subsequently. The concentrations of both Asp and Asn did not show any clear tendency during the reaction. The differences in the concentrations of Glu, Gln, Asp, and Asn after reaction were 343, 302, 80.8, and 78.1 mg/100 g, respectively. With regard to the basic amino acids, all amino acids were produced during the reaction and the increases in Arg, Lys, and His were 462, 312, and 171 mg/100 g, respectively (**Figure 3**). Among aromatic amino acids, the increases in Phe and Tyr were marked, whereas Trp increased to only 67 mg/100 g at the end of the reaction (**Figure 4**). In the case of other α-amino acids, the concentration of Pro reached 323 mg/100 g (**Figure 5**). Ser, Thr, and Met gradually increased, and the concentrations reached were 185, 153, and 101 mg/100 g, respectively. Unlike α-amino acids, the concentration

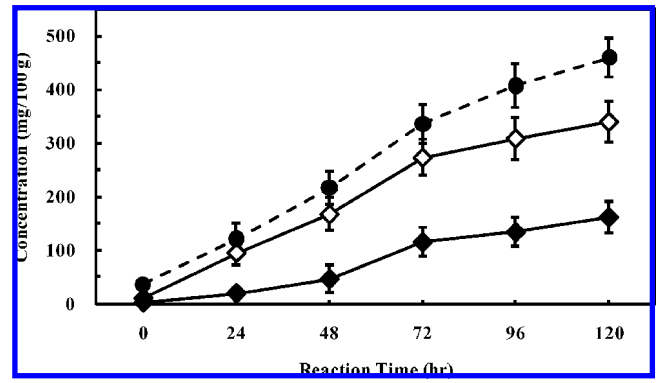

Figure 3. Time-dependent changes of basic amino acid concentrations produced from the byproduct fraction of Fuku: ◇, Lys; ●, Arg; ◆, His.

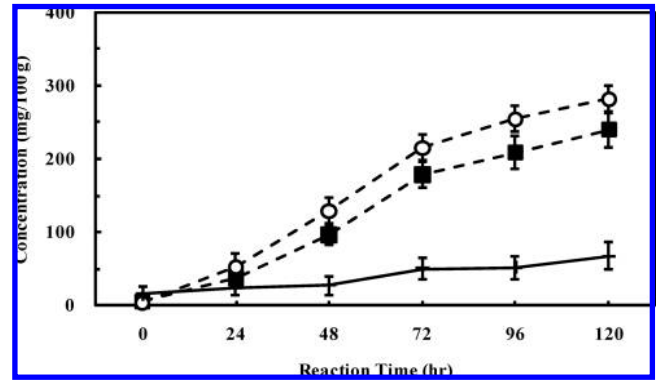

Figure 4. Time-dependent changes of aromatic amino acid concentrations produced from the byproduct fraction of Fuku: ■, Tyr; +, Trp; ○, Phe.

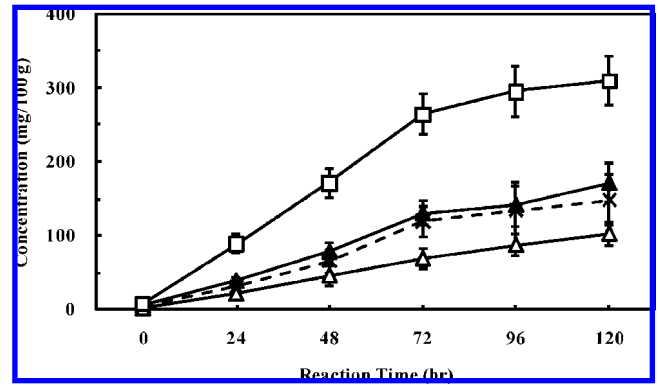

Figure 5. Time-dependent changes of sulfur, hydroxyl, and cyclic amino acid concentrations produced from the byproduct fraction of Fuku: △, Met; ▲, Ser; ×, Thr; □, Pro.

of GABA reached 148 mg/100 g in the first 24 h, but its production almost ceased thereafter (**Figure 6**). The decrease of Glu at the beginning of extraction may be attributed to the rapid conversion of Glu to GABA. Concerning the essential amino acids, all but Trp were produced at more than 100 mg from 100 g of byproduct fraction in 12 h (**Figures 1** and **3**−**5**). The yield of total α-amino acids in the 120 h reaction was about 4600 mg/100 g. Provided that the amino acids produced were attributed to proteolysis, 24−33% of the protein in byproduct fraction, which contains 14−19% protein by weight, was estimated to be degraded.

The composition of amino acids produced from the wheat-milling byproduct fraction (bran and shorts) is rich in BCAA, Arg, Lys, Gln, Phe, and GABA. The amino acid enriched extract can be used for improving mild hypertension by the effect of

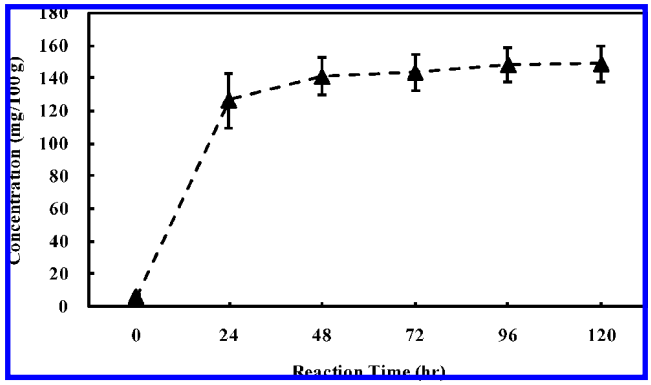

Figure 6. Time-dependent changes of GABA concentrations produced from the byproduct fraction of Fuku: ▲, GABA.

GABA, the reinforcement of the liver function, strengthening muscles, and physical fatigue relief by the effect of BCAA and the supply of essential amino acids as well. Also, soluble components such as fiber, phytic acid, cinnamic acid derivatives, B vitamins, and minerals are provided together. Other than the biological benefits, an application of autodigestive reaction to the production of amino acids has the advantages of being a simple process and inexpensive costs. Such an approach would lead to effective utilization and a decrease of the excess amount of milling byproducts.

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