JOURNAL OF BACTERIOLOGY, July 1974, p. 82-91
Vol. 119, No. 1

Regulation of Neutral Protease Productivity in *Bacillus subtilis*: Transformation of High Protease Productivity

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Received for publication 26 February 1974

A transformable strain of *Bacillus subtilis* 6160, a derivative of *B. subtilis* 168, produces three kinds of casein hydrolytic enzymes (alkaline protease, neutral protease, and esterase) in a culture medium. *B. natto* IAM 1212 produces 15 to 20 times as much total proteolytic activity as does *B. subtilis*. Extracellular proteases produced by the two strains were separated into each enzyme fraction by diethylaminoethyl-Sephadex A-50 column chromatography. The difference in the total protease activities of extracellular proteases between the two strains was due to the amount of neutral protease. The ratios of neutral protease activity to alkaline protease activity (N/A) were 1.1 in *B. subtilis* 6160 and 13.0 in *B. natto* IAM 1212. Enzymological and immunological properties of alkaline protease and neutral protease obtained from the two strains were quite similar or identical, respectively. Specific activities measured by an immunological analysis of the two neutral proteases against casein were also equal. A genetic character of high protease productivity in *B. natto* IAM 1212 was transferred to *B. subtilis* 6160 by the deoxyribonucleic acid-mediated transformation. Among 73 transformants that acquired high protease productivity, 69 produced a higher amount of neutral protease and the ratios of N/A were changed to 15 to 60. Three other strains were transformed in the productivity of neutral protease and α-amylase simultaneously, and one showed considerable change in the production of alkaline protease and neutral protease. The specific activities (casein hydrolytic activities/enzyme molecules) of neutral proteases from the representative four transformants were equal to those of the two parental strains. These results suggested the presence of a specific gene(s) that participated in the productivity of neutral protease in *B. subtilis*.

A transformable strain of *Bacillus subtilis* Marburg produced at least three kinds of extracellular proteolytic enzymes into a sporulating culture medium: neutral protease, alkaline protease, and esterase (12, 17). The structure of alkaline protease known as subtilisin, produced by some strains of bacilli, has been studied in detail (10, 13, 14, 20, 21, 30). Furthermore, alkaline protease was reported to be important for spore formation (9). This enzyme is inhibited by diisopropylfluorophosphate (DFP) (5, 18, 23). Neutral protease of *B. subtilis* has also been purified and crystallized. This enzyme molecule contains one molecule of Zn (10, 22) and was inactivated by 2×10^{-4} M ethylenediaminetetraacetic acid (EDTA) (10). The third enzyme, esterase, possesses high esterolytic activity and low proteolytic activity. It is inactivated by DFP (1, 4, 12, 17).

Yamaguchi et al. (25) and Yuki (29) reported the participation of a specific regulator gene in the production of extracellular α-amylase by transformation of *B. subtilis* Marburg strains with deoxyribonucleic acid (DNA) from *B. natto* IAM 1212 and *B. subtilis* 1088. Yamaguchi et al. (manuscript in preparation) also showed that transformants which had higher productivity of extracellular proteases could be obtained. Yoneda et al. (26) reported a common regulator gene for the production of extracellular protease and α-amylase by an analysis of mutants obtained from a transformable strain of *B. subtilis* 6160.

B. natto IAM 1212 produces 15- to 20-fold-higher level of extracellular protease in total activity than does *B. subtilis* 6160. Attempts were made to characterize the transformation of *B. subtilis* 6160 protease productivity with DNA from *B. natto* IAM 1212. This paper suggests that a specific gene(s) regulates the production of neutral protease.

MATERIALS AND METHODS

Organisms. *B. subtilis* 6160 (*purB6 metB5 trp-3*) was a derivative of *B. subtilis* 168 of Burkholder and Giles (2). *B. natto* IAM 1212 (prototroph) was a stock

culture at Institute of Applied Microbiology, the University of Tokyo. *B. natto* IAM 1212 in this experiment was a streptomycin-resistant (Str^R) mutant derived from the original strain.

Media. Compositions of bouillon-yeast extract (BY) medium (8) and glutamate-citrate (GC) medium (15) were described previously. BYL and GCL medium contained 0.05% (wt/vol) soy bean lecithin in BY and GC medium, respectively.

Assay of protease activity. Hydrolytic activity of protease against casein was assayed by the modified method of Hagihara et al. (6). A 1-ml volume of 0.6% refined casein in 0.05 M phosphate buffer (pH 7.3) was mixed with 0.2 ml of an enzyme solution. After the mixture was incubated at 40 C for appropriate time, 1.0 ml of 0.11 M trichloroacetic acid containing 0.33 M acetic acid and 0.22 M sodium acetate was added, and the solution stood at room temperature for 30 min. The insoluble part of the mixture was removed by centrifugation, and the absorbance of the supernatant was measured with a spectrophotometer at 275 nm. One unit of enzyme activity was defined as that amount of enzyme which increased 0.001 in the absorbance, at 275 nm, per min at 40 C under the above conditions.

Assay of esterase activity. The esterolytic activity was assayed against *N*-carbobenzoxy-leucine *p*-nitrophenyl ester (*N*-CBZ-Leu-PNP) as a substrate. The reaction mixture contained 0.1 ml of enzyme solution and 0.2 ml of *N*-CBZ-Leu-PNP solution (0.1 mg/ml) in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5) containing 10% *N-N*-dimethyl formamide and 0.002 M calcium acetate. After an appropriate time for incubation at 30 C, 0.7 ml of 50% *N-N*-dimethyl formamide was added, and the absorbance at 420 nm was measured with a spectrophotometer. One unit of esterase activity was defined as that amount of enzyme which increased 0.01 in absorbance, at 420 nm, per min at 30 C under above conditions.

Assay of amylase activity. Amylase activity was assayed by the modified blue value method of Fuwa (3).

Procedure of transformation. A transforming DNA was prepared from *B. natto* IAM 1212 (Str^R) by the phenol-pH 9 buffer method of Saito and Miura (19). Transformation experiments were carried out as described by Yoshikawa (27).

Preparation of crude proteases. *B. subtilis* 6160 or *B. natto* IAM 1212 (Str^R) was cultured in GC medium at 30 C for 24 h. After cells were removed by centrifugation, proteases were precipitated from the culture fluids by 85% saturation with ammonium sulfate at pH 7.5. The precipitate was collected by filtration over Celite 545. The mixture of proteases and Celite 545 was suspended in 0.01 M Tris-hydrochloride buffer (pH 7.5) containing 0.002 M calcium acetate, and then exhaustively dialyzed against the same buffer. Celite was removed by filtration; the filtrate contained the crude protease fraction.

Measurement of the amount of neutral protease and alkaline protease in media. After cells were cultured for 24 h at 30 C and removed by centrifugation, 3 ml of the culture fluid was dialyzed against 0.01 M Tris-hydrochloride buffer (pH 7.5) containing 0.002 M calcium acetate. The dialyzed solution was subjected to chromatography on a diethylaminoethyl (DEAE)-Sephadex A-50 column (0.7 by 10 cm) which had been equilibrated with the same buffer as mentioned above. A 10-ml volume of the eluate was collected as the alkaline protease fraction. The column was then eluted by 0.01 M Tris-hydrochloride buffer (pH 7.5) containing 0.002 M calcium acetate and 1 M NaCl. The first 10 ml of the eluate was collected as the neutral protease fraction. Casein hydrolytic activity in the alkaline fraction was assayed at pH 9.0, and that in the neutral protease fraction was assayed at pH 7.0. The ratio of the activity in the neutral to that in the alkaline protease fraction was designated N/A.

Preparation of antisera. Rabbit antisera against alkaline protease (Nagarse, SAC, and NSI; see footnotes *b* to *d* in Table 2) and against neutral protease produced by *B. subtilis* YY-154 (26) were prepared. The method for the preparation of the sera and purification method of the neutral protease from the culture fluid of *B. subtilis* YY-154 will be published elsewhere.

Chemicals. Carlsberg and NSI alkaline protease were gifts of D. Tsuru, Nagasaki University. Nagarse was obtained from Nagase Co., Osaka, Japan. Alkaline protease (SAC, crystallized three times), produced by *B. subtilis* var. *amylosaccharitiicus*, was obtained from Seikagaku Kogyo Ltd., Tokyo, Japan. Soy bean lecithin was obtained from Tokyo Kasei Kogyo Ltd., Tokyo, Japan. DEAE-Sephadex A-50 was purchased from Pharmacia Chemical Inc., Sweden. All other chemicals were of reagent grade.

RESULTS

Characterization of extracellular proteases produced by B. subtilis 6160 (a DNA recipient strain) and B. natto IAM 1212 (a DNA donor strain). Millet (12) reported that at least three kinds of extracellular proteolytic enzymes were produced by *B. subtilis* Marburg, from which strain 6160 was derived. One of them is alkaline protease belonging to the subtilisin group. The second enzyme is neutral protease, containing zinc, and the third enzyme possesses high esterase activity.

We examined general properties of proteolytic enzymes produced by *B. subtilis* 6160 and *B. natto* IAM 1212. A crude protease solution obtained from the culture fluid of *B. subtilis* 6160 was chromatographed on a DEAE-Sephadex A-50 column which had been equilibrated with 0.01 M Tris-hydrochloride buffer (pH 7.5) containing 0.002 M calcium acetate. The column was eluted first by the same buffer and then by a linear NaCl gradient (0 to 1.0 M). The elution pattern of proteolytic activity from the column is shown in Fig. 1a. Three peaks (A, B, and C) of casein hydrolytic activity were observed.

On the other hand, when the crude protease from *B. natto* IAM 1212 was examined by DEAE-Sephadex A-50 column chromatography

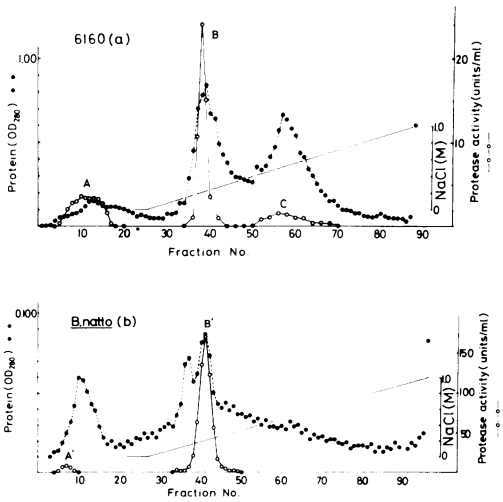

FIG. 1. *DEAE-Sephadex A-50 column chromatography of proteolytic enzymes from B. subtilis 6160 (a) and B. natto (b). The crude protease preparations obtained from the two strains were dialyzed against 0.01 M Tris-hydrochloride buffer (pH 7.5) containing 0.002 M calcium acetate and applied to DEAE-Sephadex A-50 columns (1.5 by 35 cm) which had been equilibrated with the same buffer. Elution was first with the same buffer and then with a linear gradient from 0 to 1.0 M NaCl. Absorbance at 280 nm (●) and casein hydrolytic activity (○) were monitored on each fraction.*

under the same conditions (Fig. 1b), only two peaks (A′ and B′) of the activity were observed; peak C, eluted at high concentration of NaCl in the case of *B. subtilis* 6160, could not be observed. It appeared that peak C was an esterase because it was rich in esterolytic activity toward *N*-CBZ-Leu-PNP but poor in casein hydrolytic activity. Examination by rechromatographies of the main peaks (A, A′, B, and B′) on DEAE-Sephadex A-50 columns and by polyacrylamide disc gel electrophoretic analyses at pH 8.3 and 6.5 showed that peaks A, A′, B, and B′ contained only single components in casein hydrolytic activity. Each peak, therefore, seemed to be composed of a single proteolytic enzyme.

Effects of EDTA and DFP on the four preparations were then studied in 0.01 M Tris-hydrochloride buffer (pH 7.5; Table 1). The enzymes were preincubated in 5 mM EDTA or in 1 mM DFP at 30 C for 30 min, and the remaining casein hydrolytic activity was measured. It is reported that the alkaline protease is inactivated by DFP (5, 18, 23), whereas neutral protease containing zinc is inactivated by EDTA (10, 24). The results in Table 1 showed that the peaks A and A′ corresponded to alkaline protease and that peaks B and B′ corresponded to neutral protease. This was supported further by pH optima of casein hydrolytic activity of the four preparations. The pH optimum of peaks A and A′ was 10.5 and that of peaks B and B′ was pH 7.3 (data not shown). Thus, alkaline protease was recovered in the eluate without retention on the DEAE-Sephadex A-50 column, and neutral protease was eluted from the column at a concentration of 0.2 to 0.3 M NaCl.

The properties of alkaline proteases A and A′ and those of neutral proteases B and B′ were then compared.

Thermostability of the four preparations was examined by subjecting them, in 0.01 M Tris-hydrochloride buffer (pH 7.5) containing 0.002 M calcium acetate, to various temperatures for 5 min. They were equally stable up to 55 C but almost completely inactivated by heating at 70 C for 5 min.

The effect of sodium dodecyl sulfate (SDS) in the hydrolytic activity of the four preparations is shown in Fig. 2a and b. Each preparation containing about 10 U/ml was preincubated in the presence of various concentrations of SDS at room temperature for 30 min, and the remaining hydrolytic activity was measured. The alkaline proteases A and A′ were completely inactivated in the presence of 0.2% SDS (Fig. 2a), and the neutral proteases were almost inactivated in 0.1% SDS. However, differences between A and A′ or between B and B′ were not observed.

No difference could be detected between proteases contained in A and A′ in mobilities in 75% polyacrylamide disc gel electrophoresis at pH 8.3 and 6.5 and in substrate specificities of proteolytic and esterolytic activity. No difference could also be detected in the case of B and B′ in mobilities in the gel electrophoresis and in substrate specificities.

Immunological properties of the preparations were examined by neutralization of casein hy-

TABLE 1. *Effects of EDTA and DFP on the proteolytic activity of the four preparations*[a]

Treatment	Remaining activity (%)			
	B. subtilis 6160		*B. natto* IAM 1212	
	Peak A	Peak B	Peak A′	Peak B′
None	100	100	100	100
EDTA (5 mM)	80	2	64	0
DFP (1 mM)	9	104	1	106

[a] The enzyme preparations were preincubated in the presence of 5 mM EDTA or 1 mM DFP for 30 min at 30 C, and then the remaining casein hydrolytic activities were assayed.

drolytic activity and by precipitin line formation in the double diffusion method (16). The immunological behaviors of the preparations and other alkaline proteases (Nagarse, Carlsberg, and NSI) toward rabbit antisera against alkaline proteases (Nagarse, SAC, and NSI) are summarized in Table 2. Alkaline proteases A and A' obtained from *B. subtilis* 6160 and *B. natto* IAM 1212, respectively, cross-reacted equally with antisera against Nagarse and alkaline protease (SAC) but not with antiserum against NSI. Nagarse also showed the same cross-reaction as the alkaline proteases A and A'. Carlsberg and NSI alkaline protease reacted only with antiserum against NSI. Yoshimoto et al. (28) reported that the properties of NSI alkaline protease were quite similar to those of Carlsberg alkaline protease. Neutral proteases B and B' did not cross-react with any of the antisera against alkaline proteases. The immunological behavior of the preparations toward rabbit antiserum against a neutral protease produced by *B. subtilis* YY-154 was also examined. Neutral proteases B and B' obtained from the parental strains cross-reacted equally with the serum (see Fig. 4).

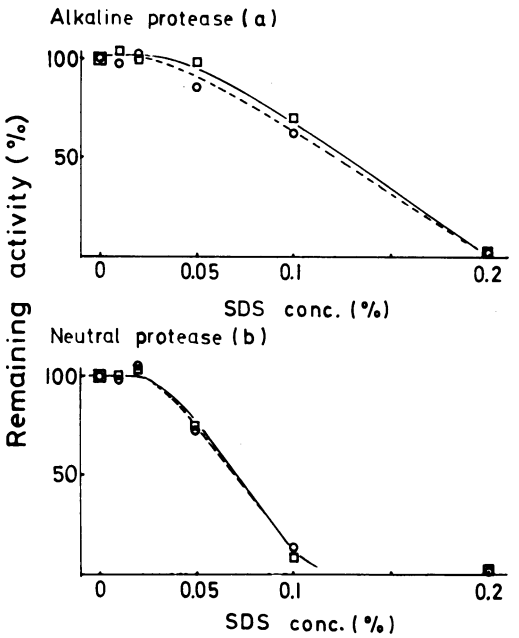

FIG. 2. *Effect of SDS on the hydrolytic activity of alkaline proteases A and A' (a) and of neutral proteases B and B' (b). Each preparation was preincubated in the presence of various concentrations of SDS at room temperature for 30 min, and the remaining casein hydrolytic activity was measured. Symbols: □, protease produced by B. natto IAM 1212; ○, protease produced by B. subtilis 6160.*

From these results, differences between alkaline and neutral protease were clearly observed, but none was found between the two alkaline proteases (A and A') and between the two neutral proteases (B and B'). Thus, the alkaline and neutral proteases from *B. subtilis* 6160 and *B. natto* IAM 1212 seemed to have the same or almost equal structure.

Transfer of high protease productivity from B. natto IAM 1212 to B. subtilis 6160. *B. subtilis* 6160 produced about 5 U of total proteolytic activity per ml in the culture fluid, whereas *B. natto* IAM 1212 produced about 100 to 120 U/ml. The difference of the protease productivity between the two strains was distinguished by "halos" around the colonies on a BY agar plate containing 1% casein (Fig. 3). Cells were inoculated on the plate and incubated at 37 C overnight. *B. natto* IAM 1212, having high protease productivity, showed a large halo around its colony, whereas a small halo was observed around a colony of *B. subtilis* 6160. NP19 and NP85, transformants having high protease productivity, also showed large halos around the colonies.

Transfer of high protease producitivity from

TABLE 2. *Immunological properties of the four protease preparations (A, A', B, and B') and alkaline proteases*

Antisera	Cross-reaction[a]						
	B. subtilis 6160		*B. natto* IAM 1212		Alkaline protease		
	Peak A	Peak B	Peak A'	Peak B'	Nagarse	Carlsberg	NSI
Anti-Nagarse serum[b]	+	−	+	−	+	−	−
Anti-SAC serum[c]	+	−	+	−	+	−	−
Anti-NSI serum[d]	−	−	−	−	−	+	+

[a] +, Cross-reacted when the preparations were examined by precipitin line formation and by neutralization of the casein hydrolytic activity; −, did not cross-react.

[b] Rabbit antiserum against Nagarse.

[c] Rabbit antiserum against alkaline protease (SAC) from *B. subtilis* var. *amylosaccharitieus*.

[d] Rabbit antiserum against NSI alkaline protease from *B. natto* strain Ns (28).

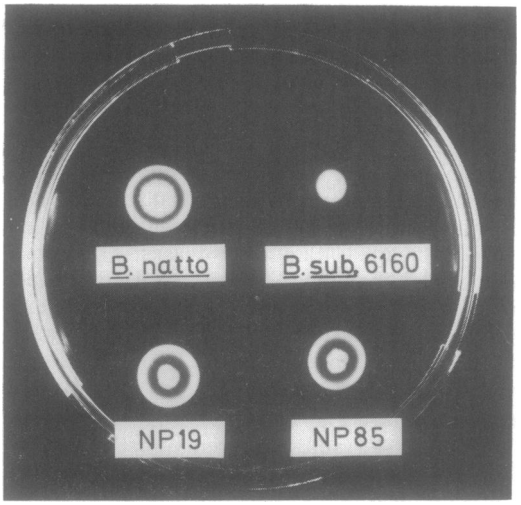

FIG. 3. *Photo of halos around the colonies on a BY agar plate containing 1% casein. B. subtilis 6160, B. natto IAM 1212, transformant NP19, and transformant NP85 are presented.*

B. natto IAM 1212 to *B. subtilis* 6160 by the DNA-mediated transformation was carried out. The cell-DNA mixture was spread on BY agar plates containing 1% casein and 100 μg of streptomycin per ml (Table 3). Eighty-seven transformants with large halos around the colonies were selected. Among them, 81 transformants having the same nutrient requirements to *B. subtilis* 6160 were isolated as transformants with high protease productivity and were used in additional experiments.

The spontaneous mutation frequency of phenotypic streptomycin resistance (Str^R) in the recipient strain was less than 10^{-8}, and the frequency of Str^R from the DNA-mediated transformation was 0.5×10^{-4}. Since a high concentration of the transforming DNA (1 μg/ml) was employed, the double transformation of Str^R and the character of phenotypic high protease productivity (Pro-sup) might be the result of two independent events. The frequency of the double transformation of Pro-sup and Str^R to the transformation of Str^R was 2 to 3%. When small amounts of DNA (0.01 and 0.001 μg/ml) were employed in the transformation, the frequencies were 0.3% and less than 0.1%, respectively.

Analysis of extracellular protease components produced by the transformants. The amounts of alkaline and neutral protease in crude protease preparations from *B. subtilis* 6160 and from *B. natto* IAM 1212 were quite different (Fig. 1a, b). Therefore, alkaline protease and neutral protease secreted into culture media by the parental strains and by the transformants were separated by DEAE-Sephadex A-50 column chromatography. Casein hydrolytic activities in the alkaline protease fraction and the neutral protease fraction were then measured. Peak C (Fig. 1a), which was rich in esterolytic activity, was included in the neutral protease fraction in this separation. Casein hydrolytic activity of peak C was so small, compared with that of neutral protease, that we believed that the proteolytic activity in the fraction was mostly due to neutral protease. We therefore assumed that the casein hydrolytic activity ratio (N/A) of neutral protease fraction to alkaline protease fraction was equivalent to the ratio of neutral and alkaline protease activity. Esterolytic activity of the neutral protease fraction against *N*-CBZ-Leu-PNP was assayed separately. Data concerning total protease activity measured at pH 7.5, alkaline and neutral protease activities, the ratio of N/A, amylase and esterase activity in culture media from the parental strains, and the transformants are summarized in Table 4.

The difference in total amount of protease activity produced by *B. natto* IAM 1212 (the DNA donor strain) and *B. subtilis* 6160 (the DNA recipient strain) was entirely due to the amount of neutral protease. Thus, the ratio N/A in *B. natto* IAM 1212 was 13.0, whereas in *B. subtilis* 6160 it was 1.1. The transformants having high protease productivity produced almost the same level of extracellular protease as did *B. natto* IAM 1212. Except for strain NP86, the amount of alkaline protease produced by the transformants was not changed. Only the amount of neutral protease was increased to the level of *B. natto* IAM 1212. Esterolytic activity against *N*-CBZ-Leu-PNP in the transformants was almost the same level as that in *B. subtilis* 6160.

TABLE 3. *Transformation of B. subtilis 6160 by a DNA from B. natto IAM 1212*[a]

Donor strain	Recipient strain	No. of transformants with selected markers:	
		str^{R}[b]	pro-sup[c]
B. natto IAM 1212 Pro-sup,[d] Str^{R}[b]	*B. subtilis* 6160 Pro^{+}, [e]Str^{S}[f]	3,930	87

[a] 1 μg of the transforming DNA per ml was employed.
[b] Phenotypical streptomycin-resistant character.
[c] Phenotypical high protease productivity in the Str^R. transformants.
[d] Phenotypical high protease productivity.
[e] Phenotypical low protease productivity.
[f] Phenotypical streptomycin-sensitive character.

TABLE 4. *Protease, amylase, and esterase activity in the culture fluids of the parental strains and the representative transformants*[a]

Strain	Protease activity (U/ml)			N/A	Amylase activity (U/ml)	Esterase activity (U/ml)
	Total	Neutral (N)	Alkaline (A)			
B. natto IAM 1212	121.6	64.9	5.0	13.0	43.3	2.0
NP 8	83.0	58.3	2.4	24.3	14.5	9.0
NP 9	89.0	67.0	3.1	21.6	13.2	10.0
NP18	85.5	61.4	2.2	27.9	14.5	7.3
NP19	91.0	54.4	2.2	24.7	17.2	8.0
NP20	89.5	57.1	2.4	23.8	11.8	9.0
NP25	82.5	60.3	1.2	50.3	13.9	8.0
NP28	74.5	62.4	3.2	19.5	13.1	8.3
NP32	79.5	71.0	2.1	33.8	15.4	9.7
NP36	77.5	52.6	1.3	32.3	11.2	6.2
NP44	79.5	69.0	2.0	34.5	12.5	8.0
NP53	75.0	61.6	2.6	23.7	12.8	7.8
NP57	76.5	63.3	1.7	37.2	12.5	7.7
NP58	102.0	66.1	1.8	26.7	12.6	5.7
NP60	104.5	99.0	1.9	52.0	12.0	10.9
NP66	78.0	72.0	2.0	36.0	13.9	8.5
NP73	84.0	62.1	1.8	34.5	12.5	6.9
NP85	79.5	71.9	1.4	51.4	11.3	7.4
NP86	80.0	53.4	7.0	7.6	12.1	5.8
NP30	103.5	74.1	2.3	32.2	46.0	11.1
NP43	107.0	101.0	2.5	40.4	43.7	14.3
NP75	84.5	70.6	2.3	30.7	39.2	9.6
B. subtilis 6160	4.9	2.4	2.2	1.1	11.9	9.5

[a] *B. subtilis* 6160 and transformants were cultured in BYL medium at 30 C for 24 h, cells were removed by centrifugation, and then enzyme activities were determined. *B. natto* IAM 1212 was cultured in GCL medium at 30 C for 24 h, cells were removed by centrifugation, and then enzyme activities were determined.

Based on these results, we classified the transformants into three groups.

1st group. Among 73 strains investigated, 69 strains were classified in this group. Seventeen typical strains in this group are shown in Table 4. The protease activities of the neutral protease fractions increased to the level of *B. natto* IAM 1212, and those of the alkaline protease fractions showed no change. Therefore, the N/A ratios were elevated from 1.1 of *B. subtilis* 6160 to 15 to 60. The amylase productivity of this group was the same as that of the recipient strain.

2nd group. Transformant NP86 was classified in this group. This strain produced about three times as much alkaline protease as did the recipient strain. The level of neutral protease in this strain was also elevated to the same level as that of *B. natto* IAM 1212 and group 1 transformants. The level of amylase activity in the culture medium showed no change.

3rd group. Transformants NP30, NP43, and NP75 were included in this group. Concerning the production of the proteases, these strains were equivalent to *B. natto* IAM 1212 and group 1 transformants. They were, however, transformed simultaneously in respect of α-amylase productivity. For the production of extracellular α-amylase, the presence of two regulator genes has been revealed by the previous experiments. One was a specific regulator gene (*amyR*) linked to the α-amylase structural gene. This was determined by the transformation of *B. subtilis* Marburg strains with DNA from *B. natto* IAM 1212 (25). The other was a common regulator gene (*pap*) for the production of α-amylase and protease. It was not linked to the α-amylase structural gene. The presence of this gene was recognized by genetic analyses of mutants obtained from *B. subtilis* 6160 (26). Since in this experiment a high concentration of transforming DNA was employed, the double transformation of high protease productivity and high α-amylase productivity might be a result of two independent events. Preliminary results showed, however, that the high α-amylase productivity of the transformants was due neither to *amyR* nor to *pap*.

Since the growth of eight strains among 81 transformants was very poor, N/A ratios in the culture media of these strains could not be investigated.

Comparison of specific activities of neutral protease produced by parental strains and transformants. Since neutral proteases obtained from *B. natto* IAM 1212 and *B. subtilis* 6160 cross-reacted with an antiserum against neutral protease (YY-154) and their casein hydrolytic activity was neutralized by the cross-reaction, specific activities of neutral proteases from the parental strains and the transformants were compared by immunological neutralization with the serum. To achieve possible bimolecular reaction with antibody, the neutralization was performed under excess of neutral protease in a very dilute solution. A 100-μliter amount of each neutral protease preparation containing about 10 U of the casein hydrolytic activity was incubated with mixtures (100 μliters) of various concentrations of the specific serum and of a control serum which had no neutralization activity against alkaline and neutral protease. The casein hydrolytic activity of each neutral protease produced by the parental strains and representative transformants (NP19 and NP58) was equally neutralized by an appropriate amount of the serum (Fig. 4). The neutralization increased linearly when the content of the antiserum against neutral protease was increased. The same neutralization profiles were observed in neutral proteases from other transformants (NP57 and NP85). These results suggest that the specific activities of the neutral proteases were identical or quite similar to each other. The antiserum against neutral protease slightly neutralized the casein hydrolytic activity of alkaline proteases obtained from *B. subtilis* 6160 and *B. natto* IAM 1212 (Fig. 4). This could not be due to the presence of anti-alkaline protease antibody because it was difficult to get antiserum against alkaline protease even if pure alkaline protease were used as an antigen.

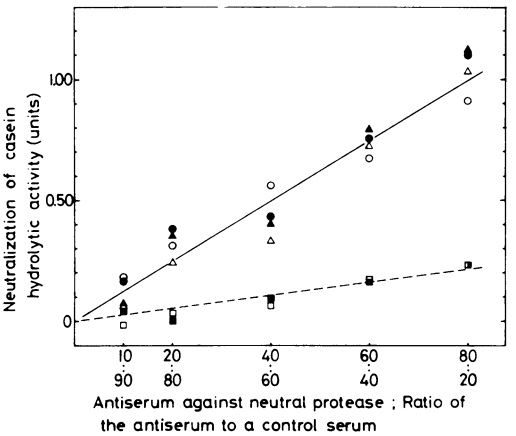

FIG. 4. *Neutralization of casein hydrolytic activity of neutral proteases obtained from B. subtilis 6160, B. natto IAM 1212, and representative transformants (NP19 and NP58) by rabbit antiserum against neutral protease (YY-154). A 100-μliter volume of each protease preparation containing about 10 U of the casein hydrolytic activity was mixed with 100 μliters of serum preparation containing various concentration of the serum against the neutral protease (YY-154). To fix protein concentration in the mixtures, a control serum which had no neutralization activity against alkaline and/or neutral protease was added in the ratios shown on the horizontal axis. The upper figure is microliters of the antiserum against neutral protease (YY-154) and lower figure is microliters of the control serum. After the mixture stood for 60 min at room temperature, the remaining casein hydrolytic activity was assayed. Symbols: ○, neutral protease from B. natto IAM 1212; ●, neutral protease from B. subtilis 6160; △, neutral protease from NP19; ▲, neutral protease from NP58; □, alkaline protease from B. natto IAM 1212; ■, alkaline protease from B. subtilis 6160. Neutral proteases from the transformants were precipitated by 85% saturation of ammonium sulfate from their culture fluids and were purified by DEAE-Sephadex A-50 column chromatography as shown in the preparation of alkaline and neutral proteases from the parental strains.*

Time courses of neutral and alkaline protease production. Time courses of the production of alkaline and neutral proteases by the parental strains and the transformants (NP19, NP57, NP58, and NP85) were examined. Figure 5 shows time courses of growth and total extracellular protease activity in *B. subtilis* 6160 and *B. natto* IAM 1212. The production of the proteases by the two strains was started when their growth reached late log phase. Total protease activity reached maximum at stationary phase. Three stages in the time course of the protease production were selected as shown by arrows in Fig. 5. Period I is late log phase of growth, i.e., maximal protease-producing phase. Period II is stationary phase, i.e., the phase when protease accumulation reached maximum. Period III is late stationary phase, i.e., the phase when cell lysis had already started and protease activity in the media was slightly reduced. The ratio of neutral protease and alkaline protease (N/A) in each stage was analyzed by the DEAE-Sephadex A-50 column chromatography (Table 5). The same experiments were performed in the four transformants (Table 5). The N/A values in every strain tested decreased from period I to period III. The reduction of the ratios suggested that the period of the production of neutral protease was earlier than that of alkaline protease.

DISCUSSION

B. natto IAM 1212 produced a large amount of extracellular protease and α-amylase. *B. subtilis* 6160, which produced a small amount of the two enzymes, was transformed by a DNA from *B. natto* IAM 1212. Eighty-one transformants having high protease productivity were isolated. They produced almost the same level of total protease activity as the DNA donor strain. Extracellular proteases produced by the parental strains and the transformants were divided into the alkaline protease fraction and the neutral protease fraction by DEAE-Sephadex A-50 column chromatography. Then the N/A ratio was calculated by casein hydrolytic activity in the fractions. The difference in the total activities of extracellular proteases produced by *B. natto* IAM 1212 and *B. subtilis* 6160 was due to the amount of neutral protease (Table 4). The high protease production in the transformants, except for NP86, was due to an elevation of the production of neutral protease.

We compared the properties of neutral and alkaline proteases produced by the DNA donor strain and the recipient strain but could not observe any difference. Thus, we assumed that alkaline protease and neutral protease produced by the two strains were the same, and tentatively named the protease structural genes *nprE* for neutral protease and *aprE* for alkaline protease.

A character of high protease productivity was transferred from *B. natto* IAM 1212 to *B. subtilis* 6160 by the DNA-mediated transformation. The character was shown as higher productivity of neutral protease in the DNA donor strain and the transformants. Since the specific activities of neutral proteases from the parental strains and the representative transformants were equal (Fig. 4), we concluded that the parental strains and the transformants should contain the same *nprE*. Therefore, the elevation of neutral protease productivity suggests the presence of a gene(s) which is concerned with the productivity of neutral protease. We may assume that the genotype of *B. natto* IAM 1212 would be *nprE* and a gene(s) for the high productivity of neutral protease and the genotype for *B. subtilis* 6160 would be *nprE* and a gene(s) for low productivity. The genetic factor(s) of the high productivity of neutral protease in the DNA donor strain is considered to be transferred to the recipient strain. To determine whether the high protease productivity is controlled by a single genetic factor or by genetic factors, we must await further experiments in which a linked marker(s) to the genetic factor(s) and/or *nprE* is used.

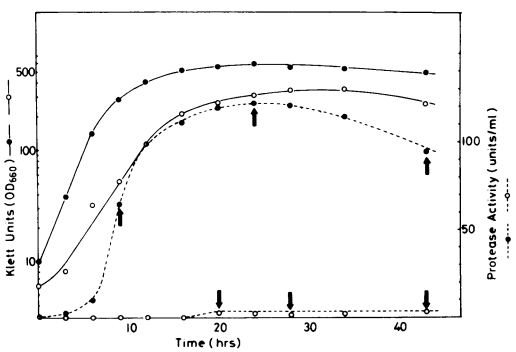

FIG. 5. *Time courses of growth and extracellular protease production by B. subtilis 6160 and B. natto IAM 1212. Cells were cultured in GCL medium at 30 C. Symbols: -●—●-, growth of B. natto IAM 1212; -○—○-, growth of B. subtilis 6160; --●---●--, protease activity in the culture medium of B. natto IAM 1212; --○---○--, protease activity in the culture medium of B. subtilis 6160. Arrows (↑) show the stages for the calculation of the N/A ratio.*

TABLE 5. *Changes in the N/A ratio during the cultures of the parental strains and four representative transformants*

Strain	N/A during culture periods:		
	I	II	III
B. natto IAM 1212	23.4	13.0	11.1
NP19	23.1	19.6	16.6
NP57	31.2	37.4	25.7
NP58	50.3	34.7	24.6
NP85	73.4	47.6	36.0
B. subtilis 6160	4.0	2.3	2.1

On the other hand, it is conceivable that some of the Str^R mutants would have pleiotropic effect resulting in altered protease production. When a high concentration of the DNA from *B. natto* IAM 1212 was employed in the transformation experiment shown in Table 3, the ratio of the numbers of Str^R and Pro-sup transformants to Str^R transformants was 2 to 3%, whereas the ratio was decreased to less than 0.1% when a small amount of the DNA was employed. This decreased ratio indicates that the Str^R and Pro-sup transformants should be derived from double transformation of independent events. Thus, almost all the transformants obtained with high protease productivity would be regulated specifically by the genetic factor(s) for the production of neutral protease. However, it is undeniable that some of the transformants might be due to the pleiotropic effect of streptomycin resistance.

Transformant NP86 produced about 20 times

as much neutral protease and about 3 times as much alkaline protease as did *B. subtilis* 6160. Compared to the elevation of the production of neutral protease in NP86, that of alkaline protease was rather small. The presence of a regulator gene(s) for the production of alkaline protease has remained obscure because the production of alkaline protease in both *B. natto* IAM 1212 and *B. subtilis* 6160 was equally small. The regulator gene(s) for high productivity of alkaline protease may be present in other strains having a high protease productivity such as *B. subtilis* var. *amylosaccharitiicus*. The strain produces a greater amount of extracellular protease than did *B. subtilis* 6160, and one-third of the protease activity was due to alkaline protease. Transformation mediated by the DNA from *B. subtilis* var. *amylosaccharitiicus* is now under investigation.

Spizizen et al. (7, 17) reported a regulator gene (*hpr*) for the production of neutral protease. The mutant having *hpr* was isolated from *B. subtilis* 168. Whether the genetic factor(s) for the high productivity of neutral protease is equivalent to *hpr* or not is now under investigation.

ACKNOWLEDGMENTS

This work was supported by grants from the Japan Foundation for Applied Enzymology and the Science Research Fund of Ministry of Education of Japan.

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