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DEMONSTRATION OF ENDO-*N*-ACETYLGLUCOSAMINIDASE AND ENDO-*N*-ACETYLMURAMIDASE AS IMPURITIES IN A CRUDE COMMERCIAL PREPARATION OF β-AMYLASE FROM BARLEY

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SUMMARY A commercial preparation of crude β-amylase from barley was unexpectedly found to solubilize cell wall peptidoglycan of *Vibrio parahaemolyticus* (A55) and the cell walls of *Micrococcus lysodeikticus* NCTC 2665. Two lytic principles were separated from the β-amylase preparation by isoelectric focusing in the range of pH 3–10. The lytic actions of these principles were elucidated by analyses of terminal groups liberated from *M. lysodeikticus* cell walls. Both the lytic principles released reducing groups but no detectable terminal amino groups, and were thus shown to be bacteriolytic hexosaminidases. Reduction of liberated reducing groups with sodium borohydride and determination of the decrease in glucosamine or muramic acid with concomitant appearance of the corresponding amino sugar alcohols as reduction products indicated that one of the lytic principles (a pI 6.8 enzyme) was an endo-*N*-acetylglucosaminidase while the other (a pI 9.5 enzyme) acted as an endo-*N*-acetylmuramidase on the cell walls of *M. lysodeikticus*. Both enzymes showed limited lytic activity against intact bacterial cell walls, but hydrolyzed the glycan moiety of cell walls of various gram-positive and gram-negative bacteria from which the non-peptidoglycan compounds had been at least partially removed.

INTRODUCTION

During studies on the cell envelope of *Vibrio parahaemolyticus* A55 it was observed that a rigid layer of the envelope, mainly composed of peptidoglycan, was solubilized by treatment with a crude preparation of barley β-amylase (crude β-amylase) which was used

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to remove polysaccharide, a "contaminant" of the rigid layer. Subsequently, it was found that this enzyme preparation also lyzed the cell walls of *Micrococcus lysodeikticus* (Tamura, 1969). The present work was on the nature of the bacteriolytic principle(s) in the crude β-amylase.

MATERIALS AND METHODS

1. *Cell walls of M. lysodeikticus*

M. lysodeikticus NCTC 2665 was cultivated with shaking in nutrient broth (pH 7.2) supplemented with 0.5% glucose. After 48 hr incubation at 37 C, cells were harvested by centrifugation and washed thoroughly with 0.85% sodium chloride solution. Cell walls were prepared from the washed cells by disruption in a Braun Mechanical Cell Homogenizer (Model MSK), followed by differential centrifugation and treatments with deoxyribonuclease, ribonuclease and trypsin (all crystalline materials) following a procedure similar to that described previously (Hamada et al., 1971). The resulting cell wall preparation was heated for 5 min at 100 C to inactivate autolysin, if present. The walls had the following amino acid and amino sugar composition in mμmoles/mg (moles/mole of total glutamic acid residues are shown in parenthesis): muramic acid 358 (0.60), glucosamine 528 (0.82), alanine 1343 (2.09), glutamic acid 641 (1.00), lysine 629 (0.98) and glycine 571 (0.89). The low apparent contents of muramic acid and glucosamine may be at least partly explained by the fact that muramic acid-6-phosphate and glucosamine-(6)-phosphate were formed during the procedure of acid hydrolysis used in analysis of the cell walls. The significance of these phosphate derivatives of amino sugars, and especially of glucosamine, will be described separately.

2. *Other cell walls and chitohexaose*

Cell walls of other organisms were prepared using a Braun Cell Homogenizer. The organisms used were *Staphylococcus aureus*, strain Copenhagen (Kato et al., 1968), *Streptococcus pyogenes*, strain S.F. 42 (Group A, type 12) (Hamada, Kotani and Kato, 1968), *Lactobacillus plantarum* ATCC 8014 (Matsuda, Kotani and Kato, 1968), and *Mycobacterium tuberculosis*, strain H37Rv (Kotani et al., 1970). Cell walls of *Corynebacterium diphtheriae*, strain Park-Williams No. 8 and *Bacillus megaterium*, strain KM were prepared by disruption of cells with a Kubota Sonic Oscillator (Model KMS-100) followed by treatment with trypsin or pronase as described previously (Kotani et al., 1959 a, b). Cell walls of *Clostridium botulinum*, strain 190L (type A) were a gift from Dr. K. Takumi, Department of Food Microbiology, Tokushima University School of Medicine (Takumi and Kawata, 1970), those of *Mycobacterium smegmatis* from Dr. I. Azuma, Department of Internal Medicine, Osaka University Medical School (Petit et al., 1969) and those of *Streptomyces fradiae* IAM 0093 and *Streptomyces lavendurae* IAM 0023 from Dr. T. Nakamura, Central Research Laboratory, Takara Shuzo Co. (Nakamura, Tamura and Arima, 1967). Envelope peptidoglycans of *V. parahaemolyticus* A55, *Escherichia coli*, strain B and a halophilic pseudomonad, strain 101 were obtained by treating the cells successively with sodium dodecyl sulfate, urea and crystalline trypsin, following the method of Kolenbrander and Ensign (1968), as modified by Tamura et al., (1969). Some of the cell wall preparations were extracted with trichloroacetic acid (TCA) or phenol to remove non-peptidoglycan compounds: with 5% TCA in the cold for cell walls of *S. pyogenes* and *C. diphtheriae*, with 5% TCA at 60 C for those of *M. tuberculosis*, and with 45% aqueous phenol at 4 C and 65 C for *L. plantarum*. However, removal of the non-peptidoglycan compounds was incomplete.

Chitohexaose was a generous gift from Dr. H. Fujio, Department of Immunochemistry, Research Institute for Microbial Diseases, Osaka University (Imanishi, Miyagawa, and Amano, 1969).

3. *Enzymes*

Crude β-amylase from barley was from Sigma Chemical Co., St. Louis, Mo., U.S.A. and hen's egg-white lysozyme (7 $\times$ crystallized) from Seikagaku Kōgyō, Tokyo. A single lot (lot No. 19b–8020) of the crude β-amylase was used throughout the present study, but another lot (lot No. 17 b–8570) has also been found to contain the lytic principle(s) for *M. lysodeikticus* cell walls.

4. *Isoelectric focusing in density gradients*

Isoelectric focusing was carried out as described by Vesterverg and Svensson (1966) and Vesterverg et al. (1967). Carrier ampholytes (Ampholine, LKB lot No. 36 LKB-Produkter AB, Bromma 1, Sweden) were used at a final concentration of 1% (v/v) to establish a pH gradient in the pH range of 3–10.

An almost linear density gradient was set up in the focusing column with sucrose, 0–50% (w/v). A specimen (320–570 mg) of the crude β-amylase was subjected to isoelectric focusing with a potential of 700 v (2–5 amp) in a 110 ml column with a cooling mantle (LKB-Produkter AB) at 1 C. After focusing for 35–46 hr, the contents of the column were collected in one ml fractions. The pH value of each fraction was immediately determined at 4 C with a pH meter (Model PHM 26, Radiometer, Denmark). The lytic activity of each fraction against the cell walls of *M. lysodeikticus*, and its β-amylase activity and absorbance at 280 mμ (OD_{280}) were measured. All determinations were made on fractions without dialysis, since there was no significant interference.

5. *Assay of cell wall lytic activity*

The standard assay system was as follows: Cell walls were suspended in deionized water at a concentration of 9 mg (dry weight)/ml. Samples of 0.5 ml of the cell wall suspension were mixed with 1.5 ml of sodium acetate buffer (pH 4.0, ionic strength 0.02) and appropriate amounts of enzyme specimens in test tubes (10 mmϕ × 100 mm) in an ice bath. The total volume was adjusted to 3 ml by supplement with deionized water. Control tubes were made by omitting either cell walls or enzymes. Tubes were incubated at 37 C and changes in absorbance at 550 mμ (OD_{550}) were recorded at 5–10 min intervals with a Shimadzu Baush & Lomb Spectronic 20 colorimeter (Shimadzu Seisakusho, Kyoto). One unit of lytic activity was defined as the amount of enzyme required to reduce the OD_{550} of the assay mixture by 50% in 30 min on incubation under the conditions described above.

6. *Chemical and analytical methods*

Reduction with sodium borohydride: The reducing groups of amino sugars were reduced by incubating cell wall lysates (dried material derived from 0.75–0.90 mg cell walls) in 0.4 ml of 0.1 M sodium borohydride solution for 20 hr at room temperature. After reduction, 0.2 ml of 0.2 M acetic acid was added to the mixture to stop the reaction, and the mixture was dried *in vacuo* over conc. sulfuric acid and solid sodium hydroxide.

Analyses for amino sugar, amino sugar alcohol and amino acid: Specimens of cell walls or their degradation products were hydrolyzed with 6 N hydrochloric acid at 100 C for 14 hr in sealed tubes, and were assayed with a Hitachi KLA-3B Amino Acid Analyzer (Hitachi Ltd., Tokyo) by a slight modification of the method of Spackman, Stein and Moore (1958). The specimens of glucosaminitol and muramicitol used as references were kindly supplied by Drs. K. Yamamoto and S. Hara, Department of Chemistry, Osaka University College of Science, respectively (Hara and Matsushima, 1966).

Other assays: Reducing sugar was determined by the method of Park and Johnson (1949). The Morgan-Elson reaction for identification of simple *N*-acetylhexosamine or disaccharide (1 → 4 linked) of *N*-acetylhexosamines was performed by the modified method described by Ghuysen, Tipper and Strominger (1966) with heating times of 7 and 30 min at 100 C. The protein content of fractions separated by isoelectric focusing was estimated on the basis of the absorbance at 280 mμ. β-amylase activity was determined by the method of Bernfeld (1955). All colorimetric determinations were carried out on a micro-scale unless otherwise stated.

7. *Paper chromatography*

One-way descending chromatography on Toyo Roshi No. 51 A filter paper was accomplished with *n*-butanol-acetic acid-water (3 : 1 : 1, by vol.) as developing solvent. Spots of *N*-acetylhexosamines and their oligosaccharides were detected by the method of Sharon and Seifer (1964), Hough et al. (1950) and Trevelyan, Procter and Harrison (1950). β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid, used as a reference, was isolated from the cell walls of *C. diphtheriae* by successive digestion with the *Chalaropsis* B enzyme and *Flavobacterium* L-11 enzyme. The procedures for isolation of this disaccharide will be described elsewhere. A specimen of β-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine isolated from *S. aureus* cell walls was generously supplied by Dr. M. Tomoeda, Department of Chemistry, Faculty of Pharmaceutical Sciences, Kanazawa University (Tipper, Tomoeda and Strominger, 1971), and was used as a reference.

RESULTS

1. *Analyses of terminal groups liberated from the cell walls of M. lysodeikticus by the action of crude barley β-amylase*

Cell walls (4.5 mg) were incubated with a

crude β-amylase (with 0.75 cell wall lytic unit) under the standard conditions and liberation of reducing sugars and terminal amino groups were measured. During incubation for 20 hr, the turbidity of the cell wall suspension decreased to a few per cent of the initial value with liberation of about 150 mμmoles of reducing groups but no significant release of terminal amino groups. The results indicate that the lytic principle(s) in the crude β-amylase preparation exerts its lytic action on *M. lysodeikticus* cell walls through the hydrolysis of glycosidic linkages in a glycan moiety of the peptidoglycan. The cell wall lysate was then assayed by the Morgan-Elson reaction as modified by Ghuysen et al. (1966) to estimate the extent of cleavage of the glycan portion. The lysate gave no appreciable reaction after either 7 or 30 min heating, indicating that it contained little or no simple *N*-acetylhexosamines and/or disaccharides (1→4 linked) of *N*-acetylhexosamines. This was confirmed by analysis of the cell wall lysate by paper chromatography. No discernible spot corresponding to that of *N*-acetylglucosamine, β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid or β-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine was detected with any of the spray reagents used.

The above findings indicate that the cell wall lytic enzyme(s) in the crude β-amylase hydrolysed the interior glycosidic linkages in the glycan moiety of *M. lysodeikticus* cell wall peptidoglycan. The latter has been shown to consist of chains of alternating β-1,4-linked residues of *N*-acetylglucosamine and *N*-acetylmuramic acid and is presumed to have an average chain length of 8–18 disaccharide units (Campbell, Leyh-Bouille and Ghuysen, 1969). To determine which reducing groups of *N*-acetylmuramyl or *N*-acetylglucosaminyl residues were cleaved, the cell wall degradation products described above were submitted to quantitative amino sugar and amino sugar alcohol analyses before and after the enzymatically liberated reducing groups of amino sugars had been reduced with sodium borohydride. Similar analyses were performed after digesting the test cell walls with hen's egg-white lysozyme. Table 1 shows that when the crude β-amylase digest of *M. lysodeikticus* cell walls was reduced with sodium borohydride the amounts of muramic acid and glucosamine residues decreased to a little more than half of their contents in the unreduced digest. In the control analysis of the digest with lysozyme, on the other hand, the amount of muramic acid residues decreased on reduction with borohydride to 54% of their content in the unreduced lysate, but the amount of glucosamine was essentially the same in reduced and unreduced specimens of the lysate.

2. *Demonstration of endo-N-acetylglucosaminidase and endo-N-acetylmuramidase in crude preparation of barley β-amylase*

The above results suggest that there may be

TABLE 1. *Comparison of the amino sugar contents in digests of M. lysodeikticus cell walls with the crude β-amylase or hen's egg-white lysozyme before and after reduction with borohydride*

Digested with	OD_{550} reduction (%)	Reducing sugars liberated (mμmoles/mg walls)	Muramic acid[a]		Glucosamine[a]	
			As prepared	After $NaBH_4$ reduction	As prepared	After $NaBH_4$ reduction
Crude β-amylase[b]	98	148	100	54	100	53
Hen's egg-white lysozyme[b]	98	120	100	54	100	107

a Data are expressed as percentages of the amount of muramic acid or glucosamine residues in the digests before reduction.

b Digestion was carried out for 20 hr under the standard assay conditions. The amounts of hen's egg-white lysozyme and crude β-amylase used were 1 and 0.75 cell wall lytic units, recpectively.

two separate lytic enzymes which attack *M. lysodeikticus* cell walls. Alternatively a less likely possibility is that the liberation of reducing groups of both glucosamine and muramic acid is due to a single *N*-acetylhexosaminidase of low substrate specificity. To examine these possibilities and separate the lytic principle(s) from the β-amylase itself, specimens of the crude β-amylase were subjected to isoelectric focusing with carrier ampholytes in the pH range of 3–10. Representative results (with 540 mg of crude β-amylase) are shown in Fig. 1 and Table 2. Two lytic principles against *M. lysodeikticus* cell walls with isoelectric points (pI) of 6.8 and 9.5, respectively were clearly separated. However the separation of the lytic principles, and especially of the pI 6.8 enzyme, from β-amylase, was not complete. About 11 and 36% of the lytic activity were recovered in the pI 6.8 fraction (tube No. 60–65) and pI 9.5 fraction (tube No. 90–98), respectively in this experiment. In seven experiments the yield of the total lytic activity varied between 25 and 46% although the recoveries of β-amylase activity and protein (measured as the OD_{280}) were invariably high (80–90%), as in the results shown in Table 2. Further, the increase in specific activity, in terms of lytic units/mg or in the ratio of lytic units/β-amylase units, was not satisfactory

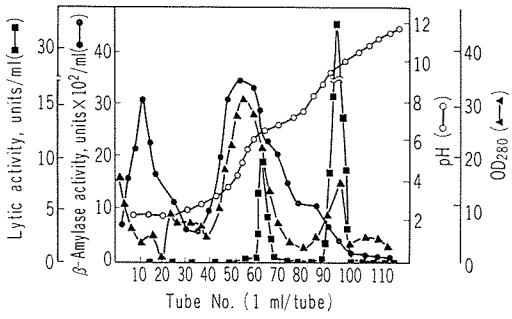

FIGURE 1. *Separation of two cell wall lytic principles in a crude preparation of barley β-amylase by isoelectric focusing in a pH gradient created by carrier ampholytes pH 3–10. A lytic activity was assayed with cell walls of M. lysodeikticus under standard assay conditions.*

(see Discussion). Active fractions (fractions in peaks of pI 6.8 and pI 9.5) were combined separately, and the pooled fractions were exhaustively dialyzed against deionized water. Then each fraction was incubated with the cell walls of *M. lysodeikticus* under standard assay conditions to determine which glycosidic bonds of *N*-acetylmuramyl or *N*-acetylglucosaminyl residues were hydrolyzed. The results are summarized in Table 3. The cell wall suspension was almost completely solubilized by incubation with either of the enzyme fractions for 24 hr. During incubations with the pI

TABLE 2. *Summary of the separation of the cell wall lytic principles in a crude preparation of barley β-amylase by isoelectric focusing in the range of pH 3–10.*

Fraction	Protein (OD_{280})	Lytic activity (units)	β-amylase (units $\times 10^3$)	$\frac{\text{Lytic activity}}{\beta\text{-amylase}} \times 10^4$	Degree of purification (relative value)
Crude β-amylase (540 mg)	1320	327	182	18.0	1.0
A pI 6.8 fraction Pooled (tube No. 60–65)	192	34.6	25.2	14.0	0.7
Peak fraction (tube No. 63)	17.6	8.3	2.6	32.0	1.8
A pI 9.5 fraction Pooled (tube No. 90–98)	149	117	8.0	150	3.1
Peak fraction (tube No. 95)	13.1	33.3	0.4	833	9.7
Other fractions (pooled)	800	tr	120	—	—

TABLE 3. *Decreases of muramic acid and glucosamine, and formation of muramicitol and glucosaminitol in cell wall digests with the pI 6.8 or 9.5 enzyme on reduction with borohydride*

Digested with	Muramic acid[a]	Glucosamine[b]	Muramicitol[a]	Glucosaminitol[b]
A pI 6.8 enzyme[c]	90	47	0	32
A pI 9.5 enzyme[c]	54	90	26	0

a Data are expressed as percentages of the amount of muramic acid residues in the digests before reduction.
b Data are expressed as percentages of the amount of glucosamine residues in the digests before reduction.
c Digestion was carried out for 20 hr under the standard assay conditions. The amounts of the pI 6.8 and 9.5 enzymes used were 0.7 and 2 cell wall lytic units, respectively.

6.8 and 9.5 fractions, respectively liberation of 142 and 190 mμmoles of reducing groups/mg cell walls and of 0.27 and 0.36 mole/mole of total glucosamine residues were observed. The reducing groups liberated by pI 6.8 fraction were from glucosamine, as evidenced by decrease of glucosamine and formation of glucosaminitol on reduction of the digest with borohydride. In contrast, the amount of muramic acid was essentially the same in reduced and unreduced samples of the digest, and no muramicitol was formed on reduction. Although there was some discrepancy between the amount of glucosamine disappearing and the amount of glucosaminitol formed, the data indicate that the pI 6.8 enzyme is an endo-*N*-acetylglucosaminidase which hydrolyzes the the glycosidic linkage of *N*-acetylglucosamine to *N*-acetylmuramic acid at a rate of one linkage per two to three glycosidic linkages of *N*-acetylglucosamine residues. By similar reasoning, it was concluded from the data in Table 3 that the pI 9.5 enzyme is an endo-*N*-acetylmuramidase which cleaves one half to one third of the glycosidic bonds of *N*-acetylmuramic acid to *N*-acetylglucosamine in the glycan moiety of *M. lysodeikticus* cell walls.

3. *A preliminary study of the properties of the pI 6.8 enzyme (endo-N-acetylglucosaminidase) and the pI 9.5 enzyme (endo-N-acetylmuramidase)*

Effect of pH on lytic activity: The lytic activity of the enzyme preparations were assayed under standard conditions except that buffers of various pH ranges were added, as shown in Fig. 2. The ionic strength of the assay system was adjusted to $\mu=0.01$ over the whole pH-range examined by addition of appropriate amounts of sodium chloride. The lytic activities of the pI 6.8 enzyme, pI 9.5 enzyme and hen's egg-white lysozyme as a function of pH are shown in Fig. 2. All three test enzymes exhibited the maximum lytic activity at about pH 4, but the optimum pH range of the pI 6.8 enzyme was significantly narrower than those of the pI 9.5 enzyme and lysozyme.

Effect of ionic strength: This was examined

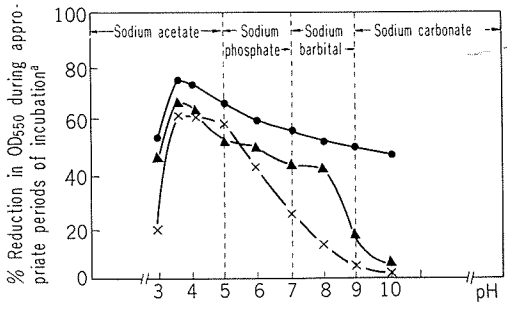

FIGURE 2. *Lytic activities of the pI 6.8 enzyme, pI 9.5 enzyme and hen's egg-white lysozyme as functions of the pH at an ionic strength of 0.01.*

pI 6.8 enzyme (0.02 *unit*, ×——×), *pI 9.5 enzyme* (*0.15 unit*, ▲——▲) *and hen's egg-white lysozyme* (*1.0 unit*, ●——●). *Assays were done under standard conditions except that buffers* ($\mu=0.01$) *of various pH ranges were used. a: The incubation period were 120, 30 and 15 min with the pI 6.8 enzyme, pI 9.5 enzyme and hen's egg-white lysozyme, respectively.*

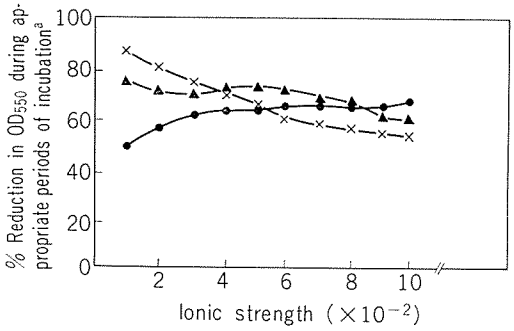

FIGURE 3. *Lytic activities of the pI 6.8 enzyme, pI 9.5 enzyme and hen's egg-white lysozyme as functions of ionic strength at pH 4.0.*

pI 6.8 enzyme (0.25 unit, ×——×*), pI 9.5 enzyme (2.5 unit,* ▲——▲*) and hen's egg-white lysozyme (1 unit,* ●——●*). Assays were made under standard conditions except that the concentration of sodium acetate buffer, pH 4.0 was varied. a: The incubation period were 240, 20 and 15 min with the pI 6.8 enzyme, pI 9.5 enzyme and hen's egg-white lysozme, respectively.*

by varying the concentration of sodium acetate buffer, pH 4.0 in the assay system. As shown in Fig. 3, the lytic activities of the pI 9.5 enzyme and hen's egg-white lysozyme were not significantly affected by ionic strength. However, a high ionic strength inhibited the lytic activity of the pI 6.8 enzyme appreciably.

Stability: The pI 6.8 and 9.5 enzymes were both completely inactivated by heating at 70 C for 15 min at neutral or weakly alkaline pH values. Storage of either enzyme preparation (as dialyzed Ampholine fractions) at 4 C for several weeks caused no significant decrease in the lytic activity against *M. lysodeikticus* cell walls.

Lytic activity range: Cell walls were obtained from a variety of gram-positive bacteria and *Streptomyces*. Their susceptibilities to the lytic actions of the pI 6.8 and 9.5 enzymes were examined with the standard assay system, by measuring reduction in turbidity of their suspensions. *C. botulinum* type A, *M. smeg-*

TABLE 4. *Lytic activity of the pI 6.8 endo-N-acetylglucosaminidase, pI 9.5 endo-N-acetylmuramidase and hen's egg-white lysozyme on cell walls from gram-positive bacteria*

Cell walls from	pI 6.8 enzyme		pI 9.5 enzyme		Hen's egg-white lysozyme	
	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr
M. lysodeikticus (NCTC 2665)	37	77	89	93	79	89
S. aureus (Copenhagen)	0	0	0	0	0	0
S. pyogenes, Group A, type 12 (S.F. 42)	0	0	0	10	0	14
L. plantarum (ATCC 8014)	11	22	7	10	18	18
B. megaterium (KM)	8	10	0	10	0	0
C. botulinum, type A (190L)	14	29	3	11	16	20
C. diphtheriae (Park-Williams No. 8)	0	0	0	0	0	0
M. tuberculosis (H37Rv)	0	0	0	0	0	0
M. smegmatis	23	35	12	40	22	26
S. fradiae (IAM 0093)	12	26	0	16	24	34
S. lavendurae (IAM 0023)	0	0	0	9	0	5

Data are expressed as percent reduction of absorbance (OD_{550}). No significant change in turbidity was recorded as 0.
The amounts of the pI 6.8 enzyme, pI 9.5 enzyme and hen's egg-white lysozyme used were 0.17, 0.7 and 1.0 cell wall lytic units, respectively.
Incubations were carried out under standard assay conditions for the times indicated.

matis and *S. fradiae* as well as *M. lysodeikticus* were among the organisms whose cell walls were more or less susceptible to the lytic actions of both enzymes (Table 4). These cell walls were also found to be susceptible to hen's egg-white lysozyme. Cell walls which were resistant to lysozyme were also refractory to both enzyme. Results of another experiment on the activity range of the pI 6.8 and 9.5 enzymes are given in Table 5. This experiment was on the susceptibilities of envelope peptidoglycans of some gram-negative bacteria and "modified" cell walls of some gram-positive organisms (after partial removal of non-peptidoglycan components) in terms of liberation of reducing groups. The assay was made under standard conditions except that the substrate concentration was reduced from 4.5 mg/3 ml to 1 mg/3 ml. Table 5 shows that glycosidic linkages of the envelope peptidoglycans of *E. coli* and a halophilic pseudomonad as well as *V. parahaemolyticus* were effectively hydrolyzed by the pI 9.5 enzyme while the pI 6.8 enzyme was active against the peptidoglycans of a halophilic pseudomonad and *V. parahaemolyticus*, but caused no significant hydrolysis of *E. coli* peptidoglycan. It is noteworthy that peptidoglycan of the halophilic pseudomonad was resistant to hen's egg-white lysozyme but was extensively hydrolyzed by both the test enzymes. Data also show that the cell walls of *S. pyogenes*, *C. diphtheriae*, *M. tuberculosis* and *L. plantarum* in the native state, were practically insensitive to the lytic actions of the test enzymes, but became susceptible on re-

TABLE 5. *Hydrolysis of the glycan moiety of cell wall peptidoglycans from the envelopes of some gram-negative and gram-positive bacteria and chitohexaose by the pI 6.8 endo-N-acetylglucosaminidase, pI 9.5 endo-N-acetylmuramidase and hen's egg-white lysozyme*

Peptidoglycan	pI 6.8 enzyme		pI 9.5 enzyme		Hen's egg-white lysozyme		Total glucosamine residues (mμmoles/mg)
	2 hr[a]	24 hr[a]	2 hr	24 hr	2 hr	24 hr	
Experiment 1							
E. coli (strain B)	0	8	168	197	41	65	626
V. parahaemolyticus (A55)	42	53	50	50	25	53	204
Halophilic pseudomonad (strain 101)	0	154	508	617	0	2	488
Chitohexaose		1500 (72 hr)[b]		1200 (72 hr)		900 (72 hr)	4914
Experiment 2							
L. plantarum (ATCC 8014)	38	106	0	22	18	64	588
C. diphtheriae (Park-Williams No. 8)	68	128	108	322	42	188	310
M. tuberculosis (H37Rv)	0	104	72	226	18	78	N.D.[c]
S. pyogenes, group A, type 12 (S.F. 42)	0	54	64	132	12	22	758

Data are expressed as amounts (mμmoles/mg substrate) of reducing sugar liberated under standard assay conditions except that the concentration of substrate was decreased to 1 mg/3 ml of assay system. The amounts of pH 6.8 enzyme, pI 9.5 enzyme and hen's egg-white lysozyme used were 0.17, 0.70, and 1.0 cell wall lytic units, respectively, in experiment 1, and 0.12, 0.58 and 0.5 units, respectively, in experiment 2.

a Incubation time

b The incubation time was longer than in other assays.

c Not determined.

moval (at least partially) of their non-peptidoglycan components. Further, chitohexaose was found to be a good substrate for both enzymes.

DISCUSSION

A number of bacteriolytic enzymes from a variety of sources have been reported to lyse different bacteria by hydrolysis of a glycan moiety of cell wall peptidoglycans. Literature on this subjects has been thoroughly surveyed in papers of Wadström and his coworkers (Wadström and Hisatsune, 1970 a, b; Wadström, 1970; Wadström and Vesterverg, 1971). The glycan moiety may be hydrolyzed enzymatically at two different sites, involving the glycosidic linkages of *N*-acetylmuramyl or *N*-acetylglucosaminyl residues. Hen's egg-white lysozyme and numerous other endo-*N*-acetylmuramidases from plant, animal and microbial sources including bacteriophages cleave only the glycosidic bonds of *N*-acetylmuramyl residues, leaving the second type of glycosidic linkage intact. A turnip lysozyme reported recently (Bernier et al., 1971; Hara and Matsushima, 1971) also belongs to this group of bacteriolytic enzymes. Thus, endo-*N*-acetylmuramidases are widely distributed in nature. In contrast, endo-*N*-acetylglucosaminidases, which attack only *N*-acetylglucosaminyl groups, seem to be rare, and only a few enzymes of this type have so far been reported. These are streptococcal muralysin, a phage-associated lytic enzyme reported by Barkulis et al. (1964), lysostaphin-glycosidase produced by a strain of *Staphylococcus epidermidis* (Browder et al., 1965), phospholipase C-associated endo-*N*-acetylglucosaminidase from *Clostridium perfringens* (Martin and Kemper, 1970), endo-*N*-acetylglucosaminidase produced by *S. aureus* strain M18 (Wadström and Hisatsune, 1970 a, b; Wadström, 1970; Wadström and Vesterberg, 1971) and endo-*N*-acetylglucosaminidase as one of autolytic enzymes of *C. botulinum* type A (Kawata and Takumi, 1971; Takumi, Kawata and Hisatsune, 1971). Among them, the one produced by *S. aureus* strain M18 has been most extensively investigated and its enzymic and physical characteristics were fairly well defined. It is remarkable that all the endo-*N*-acetylglucosaminidases hitherto reported are of bacterial origin.

The present study clearly shows that the crude commercial preparation of β-amylase from barley was contaminated by two lytic enzymes which attacked the glycosidic linkages of the cell wall peptidoglycans of some gram-positive and gram-negative bacteria. These lytic enzymes were clearly separated by fractionation of the crude β-amylase by isoelectric focusing in a gradient of pH 3–10. One of these was an endo-*N*-acetylglucosaminidase with a pI of 6.8 and the other was an endo-*N*-acetylmuramidase with a pI of 9.5. As far as we are aware, no endo-*N*-acetylglucosaminidase has previously been found in higher plants, although endo-*N*-acetylmuramidases are widely distributed in plants. The present investigation is the first on an endo-*N*-acetylglucosaminidase from a plant source, though the existence of a bacteriolytic element active on *M. lysodeikticus* in turnip was demonstrated as early as 1922 by Fleming in his classic work on " lysozyme ". A similar lytic enzyme(s) was shown to be very frequently present in the various parts of a number of flowering plants (Fleming, 1932) and in cabbages and cauliflowers as well as turnips (Thompson, 1940). Some of these bacteriolytic principles might not be a lysozyme in the strict sense of the word but in reality an endo-*N*-acetylglucosaminidase. It should be pointed out, here, that separation of the endo-*N*-acetylglucosaminidase from β-amylase was not complete, and the extent of purification achieved in terms of increase in specific activity was not satisfactory. Further, the recovery of cell wall lytic activity on isoelectric focusing was usually rather low. This low recovery might be explained by supposing that the actions of the pI 6.8 and 9.5 enzymes are synergistic, or that some co-factor(s) potentiating the lytic activity was lost during fractionation. The endo-*N*-acetylglucosaminidase and endo-

N-acetylmuramidase reported here can readily be obtained from crude preparations of barley β-amylase. They are fairly stable, unlike most bacterial endo-*N*-acetylglucosaminidases that are known to be unstable in the absence of appropriate stabilizers (Wadström, 1970).

Further work on the purification and characterization of the endo-*N*-acetylhexosaminidases in the crude β-amylase is in progress. Attempts are being made to elucidate precisely how and what extent the glycosidic linkages of *N*-acetylglucosaminyl or *N*-acetylmuramyl residues in the cell wall peptidoglycan are cleaved by these endo-*N*-acetylhexosaminidases, by analysing glycan fragments isolated from the digests of *M. lysodeikticus* cell walls after successive treatment with the pI 6.8 endo-*N*-acetylglucosaminidase or pI 9.5 endo-*N*-acetylmuramidase and with *Flavobacterium* L-11 enzyme with *N*-acetylmuramyl-L-alanine amidase activity.

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