BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 779–783 (1997)
ARTICLE NO. RC977389

Five Crucial Carboxyl Residues of 1,2-α-Mannosidase from *Aspergillus saitoi* (*A. phoenicis*), a Food Microorganism, Are Identified by Site-Directed Mutagenesis

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Received August 10, 1997

An acidic 1,2-α-mannosidase from fungus, *Aspergillus saitoi* (now designated *Aspergillus phoenicis*), is highly specific for 1,2-α-mannosidic linkage in the high-mannose type oligosaccharide at pH 5.0. The predicted amino acid sequence of several peptide regions, including aspartic acid and glutamic acid, bears striking similarities to 1,2-α-mannosidases from fungi, yeast and mouse. Active site determination of the enzyme expressed in *Saccharomyces cerevisiae* cells was performed by site-directed mutagenesis. Substitutions of Asp-269 to Glu and of the Glu-residues, Glu-273, Glu-411, Glu-414 and Glu-474, to Asp altered the drastic decrease of specific activities with Man α 1-2Man-OMe and Man_9-$GlcNAc_2$-PA as substrates and shifted the optimal pH of the mutant enzymes. From the present results, Asp-269 is probably in the ionized COO^- form, whereas one of four glutamic acid residues, probably Glu-411, is the un-ionized COOH form according to the analogy of a plausible mechanism for lysozyme catalysis. It is assumed that three glutamic acid residues, Glu-273, Glu-414, and Glu-474, are probably binding sites of substrate.

The importance of α-mannosidase (EC 3.2.1.24) in the processing system of glycoproteins in higher eukaryotic cells is well known (7-11). In the "Enzyme Nomenclature" (12), three types of α-mannosidases viz, α-D-mannoside mannohydrolase (EC 3.2.1.24), 1,2-α-mannosyl-oligosaccharide α-D-mannohydrolase (EC 3.2.1.113) and 1,3-(1,6)-mannosyl-oligosaccharide α-D-mannohydrolase (EC 3.2.1.114), are given as systematic names. There are no studies on the active site determination of α-D-mannosidases either by site-directed mutagenesis or by chemical modification.

The predicted amino acid sequence of the 1,2-α-D-mannosidase from *Aspergillus saitoi* (5) is 70%, 26%, and 35% identical with those of *Penicillium citrinum* 1,2-α-D-mannosidase (13), yeast *Saccharomyces cerevisiae* Man_9-specific α-mannosidase (14) and mouse Golgi 1,2-α-mannosidase (15), respectively. The amino acid sequence of several peptide regions, including aspartic acid and glutamic acid, in 1,2-α-mannosidases from the fungi *A. saitoi* (5), *P. citrinum* (13), and the yeast *S. cerevisiae* (14), and mammalian mouse (15), bears striking similarities with each other. This finding may indicate that the mannosidases are coded for by evolutionarily related genes at the enzymatic level. In this paper, site-directed mutagenesis of the 1,2-α-D-mannosidase gene on the expression vector, pGAM1 (5), was performed to determine the functional role of catalytic residues in the 1,2-α-D-mannosidase from *A. saitoi* expressed in yeast cells.

MATERIALS AND METHODS

The nucleotide sequence of the 1,2-α-D-mannosidase gene (D49827), *msdS*, described earlier (5) was corrected from GTTAT (from No.1387 to1391) to CGGTA; and the putative 1,2-α-D-mannosidase sequence from No. 463 to No. 464 from *A. saitoi* was corrected from Val-Ile to Arg-Tyr.

Site-directed mutagenesis. Site-directed mutagenesis of the 1,2-α-mannosidase gene was performed by the method of Kunkel *et al.* (16) using a total of twenty oligonucleotides. The following mutagenic primers were used: Asp112Ala, 5′-GTGGGCAAGATT-GcTTAtTCgAAGACAAACACCA-3′ (position in *msdS*, 322-355); Asp112Asn, 5′-GGCAAGATTaATTACTCTA-3′ (325-343); Glu124-Asp. 5′-GCCTCTTCGAcACCACCATC-3′ (362-381); Glu124Gln, 5′-GAGCCTCTTCcAGACCACC-3′ (360-378); Glu207Asp, 5′-GACGGGTACACTcGCGCTGGAcTGGACGCGCCTG-3′ (600-633);

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0006-291X/97 $25.00

Glu207Gln, 5′-GACGGGTACACTcGCGCTGcAATGGACGCGCC-3′ (600-631); Asp269Asn, 5′-GAATGGCGGTGATaACTCTTA-CTAC-3′ (792-816); Asp269Glu, 5′-GCGGTGATGAgTCTTACT-ACGA-3′ (797-818); Glu273Asp, 5′-TTACTACGAcTACCTCAT-CAAG-3′ (810-831); Glu273Gln, 5′-GACTCTTACTACcAGTAC-CTCATC-3′ (805-828); Glu377Gln, 5′-ATTGGTCCGcAGTCGTT-CAG-3′ (1120-1139); Glu411Asp, 5′-ATTCTGCGCCCGGAcGTG-ATTGAgAGCTTCTACTAT-3′ (1219-1254); Glu411Gln, 5′-ATT-CTGCGCCCGcAAGTGATTGAgAGCTTCTACTAT-3′ (1219-1254); Glu414Asp, 5′-GAAGTGATTGAcAGCTTCTAC-3′ (1231-1251); Glu414Gln, 5′-CCGGAAGTGATTcAgAGCTTCTACTATG-3′ (1228-1255); Asp454Asn, 5′-TCAGGTCTGACGaACGTtAACGCGGCC-AAC-3′ (1348-1377); Glu474Asp, 5′-CTGTTTGCCGAcGTGATG-AAA-3′ (1411-1431); Glu474Gln, 5′-CCTGTTTGCCcAGGTGAT-GAAATAC-3′ (1410-1434); Asp486Asn, 5′-GTTTGCGGAAaA-cGCGGCCTGGC-3′ (1446-1468); Glu504Gln, 5′-GTGTTCAAC-ACCcAGGCACATCCAGTaCGcGTGAGTAGTACA-3′ (1498-1539). Mismatches with the original sequence of the *msdS* are indicated in lower case. The position of oligonucleotides in parentheses is numbered according to the *msdS* sequence in ref. (5). The mutation was verified by DNA sequencing before subcloning the gene into the expression vector pGAM1. The plasmid pGAM1 containing wild type or mutated *msdS* was transfected into *S. cerevisiae* YPH250 strain (*MAT*a, *ura3, trp1, his3* and *leu2*) and each transformant was grown in the minimum medium (0.5% casamino acids, 0.67% yeast nitrogen base w/o amino acids, 2% glucose, 0.004% adenine hemisulfate salt, 0.002% uracil) at 30°C. The supernatant from recombinant yeast culture was dialyzed against 10 mM sodium acetate buffer, pH 5.0, and used for 1,2-α-mannosidase assay.

Assays. When Manα1-2Man-OMe was used as a substrate, the enzyme was incubated with 20 μg of the substrate in 15 mM sodium acetate buffer, pH 5.0, at 37°C, then released mannose was determined by the method of Somogyi (17). When $Man_9GlcNAc_2$-PA (pyridylaminated high-mannose type oligosaccharide, purchased from Takara Biomedicals Co., Japan) was the substrate at 10pmol, each reaction mixture was incubated in 50 mM sodium acetate buffer, pH 5.0, at 37°C. The reaction product was analyzed on HPLC (Amide-80 column) under the conditions described by Hase *et al.* (18), and increases in the peak area of digested oligosaccharides were measured. For both assays, one katal of 1,2-α-mannosidase was defined as the amount of enzyme required to liberate 1 mol of mannose per s at 37°C and pH 5.0.

Deglycosylation and immunoblotting. Culture supernatants (950 μl) of the yeast transformants having wild type or mutant *msdS* were treated with trichloroacetic acid (TCA) and centrifuged. The pellets were dissolved in 100 mM sodium phosphate buffer, pH 8.0, containing 0.01% SDS and heat denatured. After deglycosylation by *N*-glycanase at 37°C for 48 h, the proteins were separated by SDS-PAGE as described by Laemmli (19). The proteins were detected by immunoblotting using anti-*Aspergillus* 1,2-α-mannosidase antibody (raised in rabbit) and peroxidase-conjugated anti-rabbit IgG secondary antibody.

Determination of 1,2-α-mannosidase protein. To determine the protein for 1,2-α-mannosidase, culture supernatant from the yeast transformant was dot-blotted onto PVDF membrane and treated with anti-1,2-α-mannosidase antibody. The protein-antibody complexes were detected employing the ECL system (Amersham) and fluorography, then the intensity of the protein signal was measured using a ScanJet 4c scanner (Hewlett Packard) and a BioMax 1D image analysis system (Kodak). Bovine serum albumin (BSA) and anti-BSA antibody raised in rabbit were used for the standard.

RESULTS AND DISCUSSION

Several specific mutations were introduced by site-directed mutagenesis in the predicted active sites and substrate-binding regions of the enzyme. The mutations were selected on the basis of sequence comparison of the enzyme (5) with other 1,2-α-D-mannosidases from *P. citrinum* (13), yeast (14) and mouse (15). Mutations were focused at positions Asp-112, Glu-124, Glu-207, Asp-269, Glu273, Glu-377, Glu-411, Glu-414, Asp-454, Glu-474, Asp-486, and Glu-504. These amino acid residues were highly conserved in the enzymes from fungi (5, 13), yeast (14) and mouse (15).

TABLE 1

The Activities of Wild Type and Mutant Enzymes Expressed in *Saccharomyces cerevisiae* Cells for the Hydrolyses of Manα1-2Man-OMe and $Man_9GlcNAc_2$-PA at pH 5.0

Enzyme	Relative activity (%)[a] with Manα1-2Man-OMe	Relative activity (%)[a] with $Man_9GlcNAc_2$-PA
Wild type	100	100
Asp112Ala	7	—[b]
Asp112Asn	27	—
Glu124Gln	0	1.1
Glu207Gln	0	1.1
Asp269Asn	0	0
Glu273Gln	0	0
Glu377Gln	24	—
Glu411Gln	0	0
Glu414Gln	0	0.3
Asp454Asn	73	—
Glu474Gln	0	0
Asp486Asn	24	—
Glu504Gln	34	—

[a] Katals in a unit volume of culture supernatants were compared and expressed as the relative activity.
[b] —, not examined.

Substitutions of Glu-124, Glu-207, Glu-273, Glu-411, Glu-414, or Glu-474 for Gln resulted in mutant enzymes exhibiting complete loss of the activity for Manα1-2Man-OMe hydrolysis (Table 1). Substitution of Asp-269 for Asn in mutant enzymes also resulted in complete loss of the activity for Manα1-2Man-OMe and $Man_9GlcNAc_2$-PA hydrolysis (Table 1). Western blot analysis of yeast cell extracts digested by *N*-glycanase showed about 50 kDa proteins reactable with rabbit anti-(*A. saitoi* 1,2-α-D-mannosidase) serum, which is consistent with the apparent molecular mass of the native enzyme (Figure 1). Furthermore, mutant enzymes of Asp269Glu, Glu273Asp, Glu414Asp, Glu414-Asp and Glu474Asp showed almost complete loss of the specific activities for Manα1-2Man-OMe and $Man_9GlcNAa_2$-PA as substrates (Table 2). Optimal pH of Asp269Glu shifted from pH 5.0 to 5.6. The shift of optimal pHs from 5.0 to 3.0 were observed by mutaions Glu273-Asp, Glu411Asp and Glu474Asp (Figure 2); the shift of pH 5.0 to 4.0 is shown in Glu414Asp. The mutants, Asp269Glu and Glu273Asp, showed only trace activities of less than 0.6% of the original for both substrates

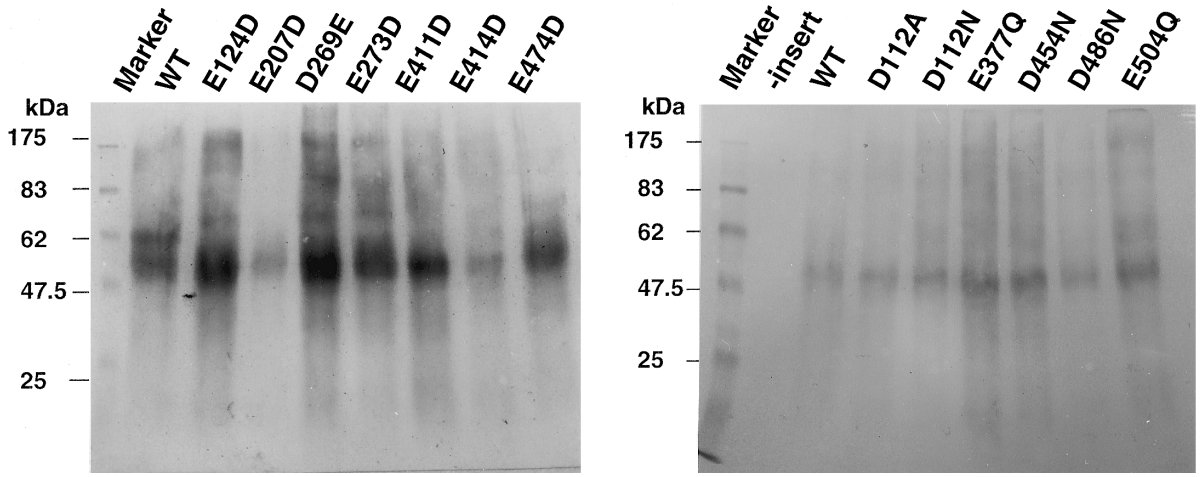

FIG. 1. Western blot analyses of the expressed proteins in *Saccharomyces cerevisiae* YPH250. Proteins were detected by using anti-*Aspergillus* 1,2-α-mannosidase antibody as described in Materials and Methods. Positions of the mutated amino acid residues are indicated above the lanes. WT; wild type.

(Table 2). These results demonstrated that the five carboxyl group residues, Asp-269, Glu-273, Glu411, Glu-414, and Glu-474, are crucial as catalytically essential residues of the 1,2-α-mannosidase from *A. saitoi*.

According to the secondary structure prediction of Chou and Fasman (20), the two crucial Asp-269 and Glu-273 residues are located in the β-turn structure, and the two glutamic acid residues, Glu-411 and Glu-414, are in the 12th α-helix from N-terminus. The prediction method also shows that Glu-474 is located in the 13th α-helix of the enzyme.

Complete loss of the activity for glycol chitin of the site-directed mutants, Glu35Asp and Asp52Glu, of lysozyme (EC 3.2.1.17) was previously reported (21,22). In the free enzyme, Asp-52 is in a very polar environment and has a nearly normal pK_a value of 3.6. In contrast, Glu-35 is in a hydrophobic environment and, probably as a consequence, has a substantially elevated pK_a value of 6.3. Hen lysozyme is maximally active at pH 5, and its catalytic activity decreases in a manner consistent with the requirement that Asp-52 be ionized and Glu-35 not, comparable to the situation in the carboxyl proteases (EC 3.4.23.-) (23).

Based on site-directed mutagenesis experiments, Totsuka and Fukazawa proposed an action mechanism of soybean β-amylase (EC 3.2.1.2) involving the

TABLE 2

Specific Activities of Wild Type and Mutant Enzymes Expressed in *Saccharomyces cerevisiae* Cells for the Hydrolyses of Manα1-2Man-OMe and $Man_9GlcNAc_2$-PA

	Specific activity			
	Manα1-2Man-OME (mkat/kg protein)	$Man_9GlcNAc_2$-PA (μkat/kg protein)		
Enzyme	pH 5	pH 5	Optimal pH	[pH]
Wild type	45.7 (100)[a]	389 (100)[a]	389 (100)[a]	[5.0]
Glu124Asp	n.d.[b]	19.0 (4.9)	106 (27.2)	[3.0]
Glu207Asp	133 (291)	957 (246)	1500 (386)	[4.0]
Asp269Glu	0.12 (0.3)	0.9 (0.2)	1.2 (0.3)	[5.6]
Glu273Asp	0.06 (0.1)	0.4 (0.1)	2.2 (0.6)	[3.0]
Glu411Asp	n.d.	0.3 (0.1)	5.8 (1.5)	[3.0]
Glu414Asp	n.d.	5.3 (1.4)	10.9 (2.8)	[4.0]
Glu474Asp	n.d.	1.1 (0.3)	15.8 (4.1)	[3.0]

Note. 1,2-α-Mannosidase activities toward Manα1-2Man-OMe or $Man_9GlcNAc_2$-PA were measured at 37°C as described in Materials and Methods. For the degradation of $Man_9GlcNAc_2$-PA, activities measured at pH 5.0 or at their optimal pH as estimated from the results shown in Figure 2 are compared. Values in the squared parentheses show the optimal pH for wild type and/or mutant 1,2-α-mannosidase.

[a] The values in parentheses denote the relative ones of specific activities.

[b] n.d., not detected.

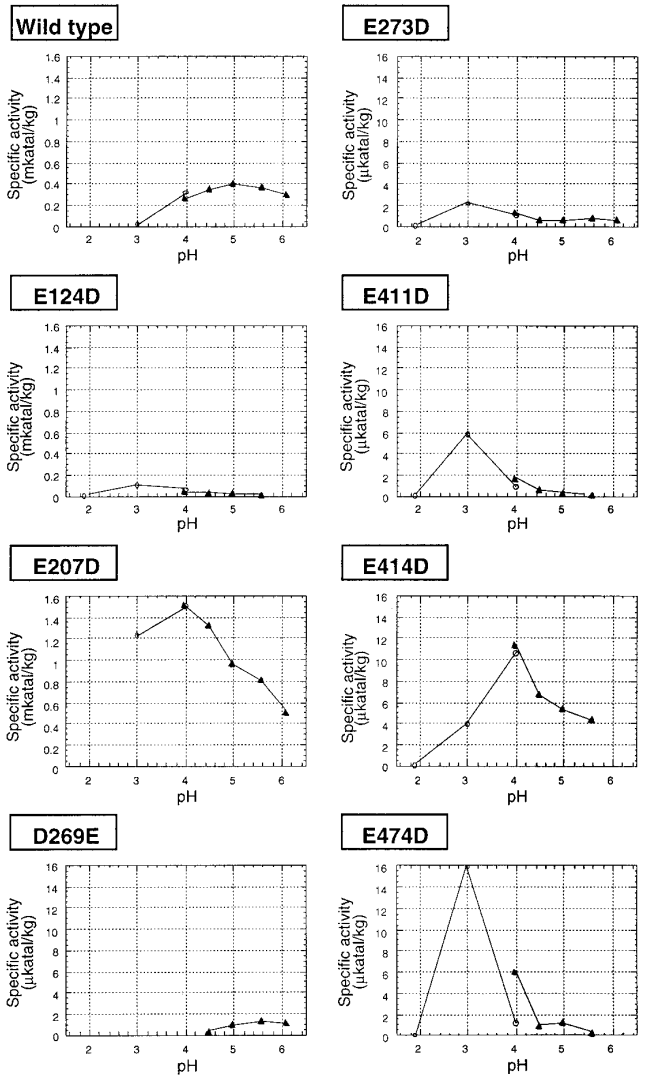

FIG. 2. Effect of pH on the specific activities of wild type and mutant enzymes for the hydrolysis of PA-sugar chain, $Man_9GlcNAc_2$-PA. Totally 10 μl of the reaction mixture containing 10 pmol of $Man_9GlcNAc_2$-PA, the dialyzed culture-supernatant of yeast, and 50 mM of sodium acetate/HCl buffer (circles) or sodium acetate/acetic acid buffer (triangles) was incubated at 37°C. The reaction was stopped by heating to 100°C, then analyzed on HPLC as described in Materials and Methods. Each activity of the enzyme was expressed as a specific activity. Open circle (○), sodium acetate-HCl buffer. Closed triangle (▲), sodium acetate-acetic acid buffer.

interactions of three essential amino acid residues (Asp-101, Glu-186 and Glu-380) in concert with Leu-388, and assumed an indispensable role for Asp-101 (24,25).

From our present results, Asp-269 is probably the ionized COO^- form, whereas one of four glutamic acid residues, probably Glu-411, is the un-ionized COOH form according to the analogy of a plausible mechanism for lysozyme catalysis (6). Nishio *et al.* (26) reported that the hydroxyl groups at C-2, -3, and -4 of the mannopyranoside of the non-reducing end were critical for the substrate recognition by non-specific jack bean α-mannosidase. It is assumed that three glutamic acid residues, Glu-273, Glu-414 and Glu-474, are probably binding sites of the enzyme for a substrate. To the authors' knowledges, this is a first paper on catalytic residues of 1,2-α-mannosidase by site-directed mutagenesis.

ACKNOWLEDGMENT

This work was supported by a grant-in aid for cooperative research from the Ministry of Education, Science and Culture of Japan.

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