APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1996, p. 3794–3799 Vol. 62, No. 10
0099-2240/96/$04.00+0
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Stereospecific Production of the Herbicide Phosphinothricin (Glufosinate): Purification of Aspartate Transaminase from *Bacillus stearothermophilus*, Cloning of the Corresponding Gene, *aspC*, and Application in a Coupled Transaminase Process

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Received 14 February 1996/Accepted 1 August 1996

We have isolated and characterized an aspartate transaminase (glutamate:oxalacetate transaminase, EC 2.6.1.1) from the thermophilic microorganism *Bacillus stearothermophilus*. The purified enzyme has a molecular mass of 40.5 kDa by sodium dodecyl sulfate gel analysis, a temperature optimum of 95°C, and a pH optimum of 8.0. The corresponding gene, *aspC*, was cloned and overexpressed in *Escherichia coli*. The recombinant glutamate:oxalacetate transaminase protein was used in immobilized form together with 4-aminobutyrate:2-ketoglutarate transaminase (EC 2.6.1.19) from *E. coli* for the production of L-phosphinothricin [L-homoalanin-4-yl-(methyl)phosphinic acid], the active ingredient of the herbicide Basta (AgrEvo GmbH), from its nonchiral 2-keto acid precursor 2-oxo-4-[(hydroxy)(methyl)phosphinoyl]butyric acid (PPO). In this new coupled process conversion rates of ca. 85% were obtained with substrate solutions containing 10% PPO by using only slight excesses of the amino donors glutamate and aspartate. The contamination of the reaction broth with amino acid by-products was <3%.

In previous publications (2, 11) we have described the stereospecific production of L-phosphinothricin [L-homoalanin-4-yl-(methyl)phosphinic acid], the active ingredient of the broad-spectrum, nonselective herbicide Basta (AgrEvo GmbH), by enzymatic transamination from its corresponding 2-oxoacid, 2-oxo-4-[(hydroxy)(methyl)phosphinoyl]butyric acid (PPO). The applied enzyme was 4-aminobutyrate:2-ketoglutarate transaminase (EC 2.6.1.19) from *Escherichia coli*. The immobilized transaminase exhibited a remarkable temperature tolerance up to 55°C and a long-term operational stability of several months.

However, a major drawback for the technical application of transamination reactions is their equilibrium constant near unity, resulting in an incomplete conversion of the keto acid to its corresponding amino acid. One possible way to overcome this limitation is by using aspartate as the amino donor. The reaction by-product oxalacetic acid decarboxylates spontaneously to pyruvic acid in aqueous solution, thus shifting the equilibrium of the reaction towards the synthesis of the desired amino acid product (18). Since the L-phosphinothricin-specific transaminase we are using in our process does not accept aspartate as an amino donor (11), the only way to take advantage of the above-described mechanism is by addition of an aspartate transaminase (glutamate:oxalacetate transaminase [GOT] [EC 2.6.1.1]) as a second enzyme in a coupled reaction (9a). In such a two-enzyme process the primary amino group donor glutamate is regenerated from aspartate as the secondary donor, thus linking the equilibrium shifting caused by the decomposition of oxalacetic acid to the L-phosphinothricin-producing reaction.

In order to identify a suitable aspartate transaminase for a technical process, we screened a number of thermophilic microorganisms for GOT activities. In this article we describe the isolation and characterization of an aspartate transaminase from *Bacillus stearothermophilus*, the cloning and overexpression of the corresponding gene, *aspC*, in *E. coli*, and the application of the GOT protein together with the L-phosphinothricin-specific transaminase in a coupled enzymatic reaction.

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MATERIALS AND METHODS

Bacterial strains, media, enzymes, and chemicals. *B. stearothermophilus* (ATCC 12980) was obtained from the American Type Culture Collection, Rockville, Md. The subcloning and expression experiments were performed with *E. coli* W3110, a derivative of the strain K-12 λ^- F^- (ATCC 27325). Plasmid-carrying transformants were grown in Luria-Bertani medium (7) with 50 μg of the appropriate antibiotic per ml. For transaminase expression the bacterial cultures at an optical density of 0.5 were induced with 1 mM isopropylthiogalactoside (IPTG), and the cells were harvested 4 h after induction.

Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, T4 DNA polynucleotide kinase, alkaline phosphatase, and the large fragment of DNA polymerase I (Klenow enzyme) were obtained from New England Biolabs Inc., Beverly, Mass., and from Boehringer GmbH, Mannheim, Germany. PCRs were carried out with *Taq* polymerase, a DNA amplification kit, and a DNA thermal cycler from Perkin-Elmer Cetus, Norwalk, Conn. The DNA sequencing reactions were performed with a sequencing kit from United States Biochemicals, Cleveland, Ohio, and Sequenase T7 polymerase. [α-^{32}P]dCTP and α-^{35}S-dATP were purchased from New England Nuclear Research Products, Dreieich, Germany. PPO was synthesized at Hoechst AG, Frankfurt, Germany. The 2-keto and amino acids used in the enzyme assays and production experiments were from Sigma, Munich, Germany. The polymer carrier VA-Epoxy Biosynth was obtained from Riedel de Haen, Hannover, Germany.

Enzyme purification. *B. stearothermophilus* was cultivated at 56°C in Luria-Bertani medium in a 10-liter fermentor. At the end of the log growth phase the cells were harvested by centrifugation and frozen in liquid nitrogen. The frozen bacteria were resuspended in 50 mM phosphate buffer containing 10 μM pyridoxal phosphate, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.0) (2.5 ml of buffer per g of frozen cells) and disrupted by sonication. The resultant slurry was centrifuged, and the supernatant was subjected to ammonium sulfate precipitation. The transaminase precipitated between 40 and 60% ammonium sulfate saturation. After dialysis against buffer A [20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 10 μM pyridoxal phosphate, 5 mM 2-mercaptoethanol (pH 7.0)] the enzyme preparation was heated (10 min, 80°C) and the precipitated proteins were removed by centrifugation. The proteins in

TABLE 1. Plasmids used and constructed in this work

Plasmid	Description	Source or reference
pUC12	Vector, Ap^r, *lac* promoter	17
pUC19	Vector, Ap^r, *lac* promoter	17
pGT2	*gabT* gene from *E. coli* on a 1.6-kb *Dra*I-*Bam*HI fragment in pMLC12	2
pTD1	*gabT* gene from *E. coli* on a 1.6-kb *Eco*RI-*Bam*HI fragment in pUC12	This work
pASP1	*aspC* gene from *B. stearothermophilus* on a 2.5-kb *Eco*RV fragment in pUC19	This work
pKOM1	*gabT* gene from *E. coli* on a 1.6-kb *Eco*RI-*Bam*HI fragment and *aspC* gene from *B. stearothermophilus* on a 1.2-kb *Pst*I-*Hin*dIII fragment in tandem orientation in pUC12	This work
pICT 212	*aspC* gene from *Bacillus* sp. strain YM-2 on a 2.5-kb *Eco*RI-*Hin*cII fragment in pUC18	13

the supernatant were placed on a Q-Sepharose column (equilibrated in buffer A), and bound proteins were eluted with a linear gradient from 0 to 0.75 M KCl in buffer A. The active fractions were pooled, concentrated by ammonium sulfate precipitation, and applied on a gel filtration column (Superdex 200; Pharmacia). The column was operated in buffer A plus 0.1 M KCl. The active fractions were pooled, precipitated by ammonium sulfate, and dialyzed against 20 mM imidazole buffer, pH 7.5. The proteins were then loaded on a PBE 94 (Pharmacia) chromatofocusing column equilibrated with the same buffer. The proteins were eluted with 12.5% Polybuffer (Pharmacia) in water, adjusted to pH 3.9. The pH of the eluted fractions was measured and readjusted immediately to pH 7.0. The transaminase was eluted at a pH of 5.0. The Polybuffer was separated from the enzyme by passing the pooled fractions over a Superdex 75 (Pharmacia) column. The purified transaminase was stored at −80°C for several months without loss of activity.

Immobilization of the transaminases. After a partial purification by ammonium sulfate precipitation, heat precipitation, and fractionation on Q-Sepharose HP both transaminases were immobilized separately to the polymer carrier VA-Epoxy Biosynth (Riedel de Haen) as described by Schulz et al. (11).

Amino acid sequencing. The first 43 N-terminal amino acids of the GOT protein were determined by gas phase sequencing with a 477a analyzer from Applied Biosystems, Inc., Foster City, Calif., by using standard procedures.

Cloning experiments and plasmid constructions. The plasmids used and constructed in this work are listed in Table 1. Genomic DNA was isolated from *B. stearothermophilus* by the procedure of Meade et al. (8). Oligonucleotide primers were synthesized by automated phosphoramidite chemistry (16) with an Applied Biosystems DNA synthesizer, model 391.

PCRs were performed in volumes of 100 μl containing 50 ng of genomic DNA and 1 μg of each primer. The amplification program was 25 cycles of 1 min at 94°C, 2 min at 37°C, and 3 min at 72°C plus 15 s at each cycle.

The *aspC* gene from *Bacillus* sp. strain YM-2 (14), which was used as a heterologous hybridization probe, was obtained on plasmid pICT212 from K. Tanizawa, Osaka University, Osaka, Japan. DNA hybridization probes were labeled with [α-^{32}P]dCTP by using the Megaprime DNA labelling kit from Amersham Buchler GmbH, Braunschweig, Germany.

The hybridization and subcloning experiments were performed by standard procedures described by Maniatis et al. (7).

Sequence and computer analyses. The nucleotide sequence of the *aspC* gene from *B. stearothermophilus* was determined for both strands by the chain termination method (10) with double-stranded DNA templates (4) and with α-^{35}S-dATP as the radioactive label.

The sequences obtained from the subcloned PCR fragments and restriction fragments were completed by the use of suitable oligonucleotide primers, which were synthesized as described above. Comparisons of DNA and protein sequences were conducted with the standard alignment programs HIBIO DNASIS and HIBIO PROSIS, distributed by Hitachi Software Engineering Co., Ltd., Yokohama, Japan, by using the EMBL sequence database compiled by the European Molecular Biology Laboratory.

Enzyme assays. The GOT from *B. stearothermophilus* was measured in crude extracts or purified enzyme preparations from wild-type bacteria or recombinant *E. coli* strains by incubating aliquots of the enzyme solution in a volume of 100 μl with 50 mM Tris-HCl (pH 8.0), 10 μM pyridoxal phosphate, 50 mM 2-ketoglutarate, and 50 mM aspartate for 30 min at 70°C. Under these conditions the temperature-sensitive GOT from *E. coli* was completely inactivated. The formation of glutamate from 2-ketoglutarate was quantified with a Biotronic LC 5001 amino acid analyzer. Protein concentrations were determined by the Coomassie brilliant blue binding assay (Bio-Rad Laboratories, Munich, Germany) of Bradford (3). Specific enzyme activities are expressed in nanokatals per milligram of protein (1 nkat = 1 nmol of turnover per s). In the substrate specificity tests 2-ketoglutarate or aspartate was replaced with other 2-keto acids or amino acids, respectively.

The phosphinothricin transaminase from *E. coli* was measured in a similar assay with a reaction mix containing 50 mM Tris-HCl (pH 8.0), 10 μM pyridoxal phosphate, 50 mM PPO, and 50 mM glutamate for 30 min at 37°C. The formation of phosphinothricin from PPO was quantified by amino acid analysis as described above.

The remaining PPO of the product solutions after enzymatic conversion was determined by ^{35}P nuclear magnetic resonance analysis with a Bruker AM 360 nuclear magnetic resonance spectrometer in the Hoechst analytical laboratory.

The optical purity of the produced phosphinothricin was measured as described by Schulz et al. (11).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to the GenBank/EMBL data bank under accession number X93600.

RESULTS

Purification and characterization of the GOT protein. Several thermophilic microorganisms were screened for thermostable GOT activities. The highest specific enzyme activity at 80°C was observed in protein crude extracts from *B. stearothermophilus*. Starting with bacteria from an 8-liter fermentation, we were able to purify 920 μg of the enzyme according to the purification protocol given in Table 2. The purified enzyme was found to be homogeneous by the criterion of sodium dodecyl sulfate-gel electrophoresis and had a molecular mass of ca. 40,500 Da (data not shown). The N-terminal amino acids of the enzyme were determined by gas phase sequencing: H_2N-Met-Lys-Leu-Ala-Lys-Arg-Val-Ala-Ser-Leu-Thr-Pro-Ser-Ala-Thr-Leu-Ala-Ile-Thr-Glu-Lys-Ala-Lys-Glu-Leu-Lys-Ala-Ala-Gly-(Arg)-Asp-Val-Ile-Gly-Leu-(Gly)-Ala-(Gly)-Glu-Pro-Asp-Phe-Asn. Amino acids in parentheses were not identified unambiguously. This sequence shows an 80% amino acid identity with the N terminus of the GOT protein from *Bacillus* sp. strain YM-2 published by Sung et al. (13).

The temperature optimum of the transaminase was 95°C; the pH optimum was 8.0. Substrate specificity of the purified enzyme was tested with 2-ketoglutarate as amino group acceptor. Highest activities were observed with aspartate and its analog cysteine sulfonic acid (Table 3). The enzyme showed low substrate affinities toward the aromatic amino acids (Trp, Phe, and Tyr) and toward methionine and cysteine. No reactivity was measured with the additional protein amino acids listed in Table 3. Enzyme activities for the amino group donors aspartate and glutamate with the amino group acceptors pyruvate and PPO were low (Table 3).

Cloning of the *aspC* gene. Several regions from the 5′ end of the published *aspC* gene sequence from *Bacillus* sp. strain YM-2 (13) with a 100% identity of the deduced amino acid sequence to the N-terminal sequence of the GOT protein from *B. stearothermophilus* (ATCC 12980) were chosen for the design of *aspC*-specific PCR primers. Additional primers were selected from highly conserved parts of the published se-

TABLE 2. Purification scheme of GOT from *B. stearothermophilus*

Purification step	Total protein (mg)	Total activity (μkat)	Sp act (nkat/mg)	Yield (%)
1. Crude extract	3,300	251	76	100
2. $(NH_4)_2SO_4$ precipitation	2,500	229	92	91.2
3. Heat precipitation	352	107	304	43
4. Q-Sepharose	157	99	631	39.4
5. Superdex 200	21.7	91.7	4,226	36.5
6. Chromatofocusing	6.5	45	6,923	17.9
7. Superdex 75	4.25	36.9	8,682	14.7

TABLE 3. Substrate specificity of GOT from *B. stearothermophilus* with 2-ketoglutarate, pyruvate, or PPO as the amino group acceptor

Amino group donor	Relative activity[a]		
	2-Ketoglutarate	Pyruvate	PPO
Aspartic acid	100	0.8	3.5
Glutamic acid		0.3	9.0
Tryptophan	10.3		
Cysteine	2.3		
Methionine	1.8		
Tyrosine	1.8		
Phenylalanine	0.8		
Other protein amino acids[b]	0		
Cysteine sulfonic acid	143.9		

[a] The specific GOT activity for aspartate with 2-ketoglutarate was assigned a value of 100.
[b] Val, Leu, Ile, Pro, Gly, Ser, Thr, Asn, Gln, Lys, Arg, and His.

quence, showing a high degree of homology to other aspartate transaminases from bacterial and mammalian sources (13). All primers synthesized were nondegenerate, and the nucleotide sequence was taken from the *Bacillus* sp. strain YM-2 gene.

With two 20-mer primers (5′GTGATTGGTCTTGGAGCT GG3′ and 5′TTTTTCGTAAATTTCATCTG3′) a 527-bp fragment of the *aspC* gene could be amplified from genomic DNA of *B. stearothermophilus*. The identity of the DNA fragment was confirmed by sequence analysis. The obtained gene probe was labeled with [α-^{32}P]dCTP and hybridized to Southern blot filters with various restriction digests of *B. stearothermophilus* genomic DNA (*Apa*I, *Bam*HI, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Kpn*I, *Sac*I, *Sau*3A, *Sma*I, *Xba*I, *Xho*I, *Eco*RI-*Sac*I, *Eco*RI-*Xho*I, *Hin*dIII-*Apa*I, *Hin*dIII-*Eco*RV, *Hin*dIII-*Kpn*I, *Hin*dIII-*Sac*I, *Hin*dIII-*Xho*I, *Sal*I-*Cla*I, *Sal*I-*Eco*RV, and *Sal*I-*Xho*I). In parallel, heterologous hybridizations were carried out with a 2.3-kb *Cla*I-*Eco*RI fragment from plasmid pICT212, containing the *aspC* gene from *Bacillus* sp. strain YM-2. The resulting hybridization signals were identical for both gene probes. A 1.4-kb *Sal*I band and a 2.5-kb *Eco*RV band were isolated from a 1% agarose gel and used for the further cloning experiments. The two fragments were ligated in the plasmid vector pUC19 and transformed into *E. coli* W3110. The resulting partial genomic libraries were screened in dot blot assays for the *aspC* gene. Minilysates were isolated from clones in pools of five, bound to nitrocellulose filters, and hybridized independently to the two gene probes. Individual clones from hybridizing pools were rescreened by the same procedure. Again, the hybridizations with both probes gave identical results. From the *Sal*I fragment library six positive clones were pulled out of 66 pools and were shown to contain an insert of the expected size (1.4 kb). From the 2.5-kb *Eco*RV fragment only one clone was obtained in 140 pools of the corresponding library.

The positive clones were tested in enzyme assays at 70°C for elevated GOT activity. At this temperature the *E. coli* endogenous GOT is inactive, and thus, only the expression of the recombinant enzyme will be determined in the assay. It turned out that only the *E. coli* clone harboring the 2.5-kb *Eco*RV fragment (plasmid pASP1 [Table 1]) produced a temperature-stable aspartate transaminase, while the transformants carrying the 1.4-kb *Sal*I fragments were inactive.

Sequence of the *aspC* gene. The 2.5-kb *Eco*RV fragment containing the complete *aspC* gene and the 1.4-kb *Sal*I fragment were sequenced in part by the chain termination method (10). New primers were synthesized step by step according to the collected sequence information. The restriction map of the sequenced region is shown in Fig. 1. The nucleotide sequence is given in Fig. 2. One long open reading frame of 1,197 nucleotides was found between nucleotide positions 350 and 1547. However, the translational start point of the gene is the second ATG at position 368, as could be demonstrated by comparing the deduced amino acid sequence (Fig. 2) with the N-terminal amino acids of the purified GOT protein determined by protein sequencing (see above).

Thus, the *aspC* gene from *B. stearothermophilus* encodes a protein of 393 amino acids with a calculated molecular mass of 43.9 kDa. The inactivity of the transformants carrying the 1.4-kb *Sal*I fragment can be explained by the *Sal*I site being present in the *aspC* structural gene (Fig. 1). The putative protein expressed from this fragment is missing 54 amino acids from the carboxy terminus of the GOT.

Sequence homologies. The amino acid similarities between the N-terminal regions of the aspartate transaminases from *B. stearothermophilus* and *Bacillus* sp. strain YM-2 are extended over the total length of the proteins (amino acid identity, 70.8%; similarity, 81.0%). For the two genes a sequence homology of 66.0% was found, calculated on the basis of identical nucleotides. However, despite the close relationship, there remain some significant differences between the two sequences: (i) the open reading frame of the *aspC* gene from *B. stearothermophilus* (1,197 bp) is 21 nucleotides longer than that of the corresponding *Bacillus* sp. strain YM-2 gene (1,176 bp), leading to a difference of 7 amino acids in the deduced protein products, and (ii) the GC content of the *B. stearothermophilus* gene of 52.7% is considerably higher than the value of 37.0% for the *Bacillus* sp. strain YM-2 gene, mostly because of third-base changes in the codon triplets.

***aspC* promoter.** Expression of the *aspC* gene from *B. stearothermophilus* in *E. coli* W3110 did not depend on the orientation of the 2.5-kb *Eco*RV fragment with respect to the *lac* promoter in the pUC19 vector. The GOT activities determined were nearly the same in the two orientations and were not inducible by IPTG. Since the expression seemed to be driven from *E. coli*-independent promoter elements, we inspected the 5′ region preceding the *aspC* structural gene for putative regulatory sequences. A comparison of this region with *E. coli* promoter motifs from the EMBL database revealed potential −35 and −10 sequences related to the σ^{70} factor binding domains (Fig. 2).

***aspC* expression constructs.** The *gabT* gene, encoding the phosphinothricin-specific transaminase from *E. coli* (2), was recloned from plasmid pGT2 (Table 1) as an *Eco*RI-*Bam*HI fragment into the vector pUC12 (plasmid pTD1 [Table 1]). The coding region of the cloned *aspC* gene from *B. stearothermophilus* was amplified by PCR and subcloned as a *Pst*I-

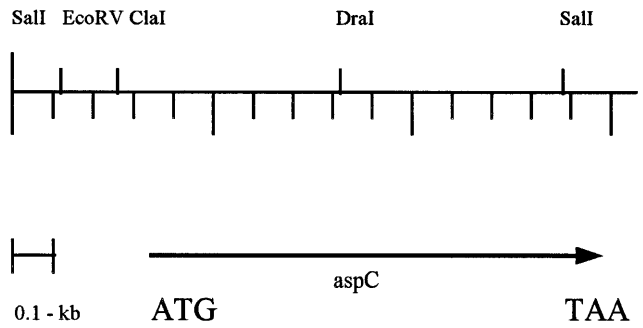

FIG. 1. Restriction map of the *aspC* region from *B. stearothermophilus*. The position and orientation of the *aspC* structural gene are indicated by the translational start and stop codons (ATG and TAA, respectively).

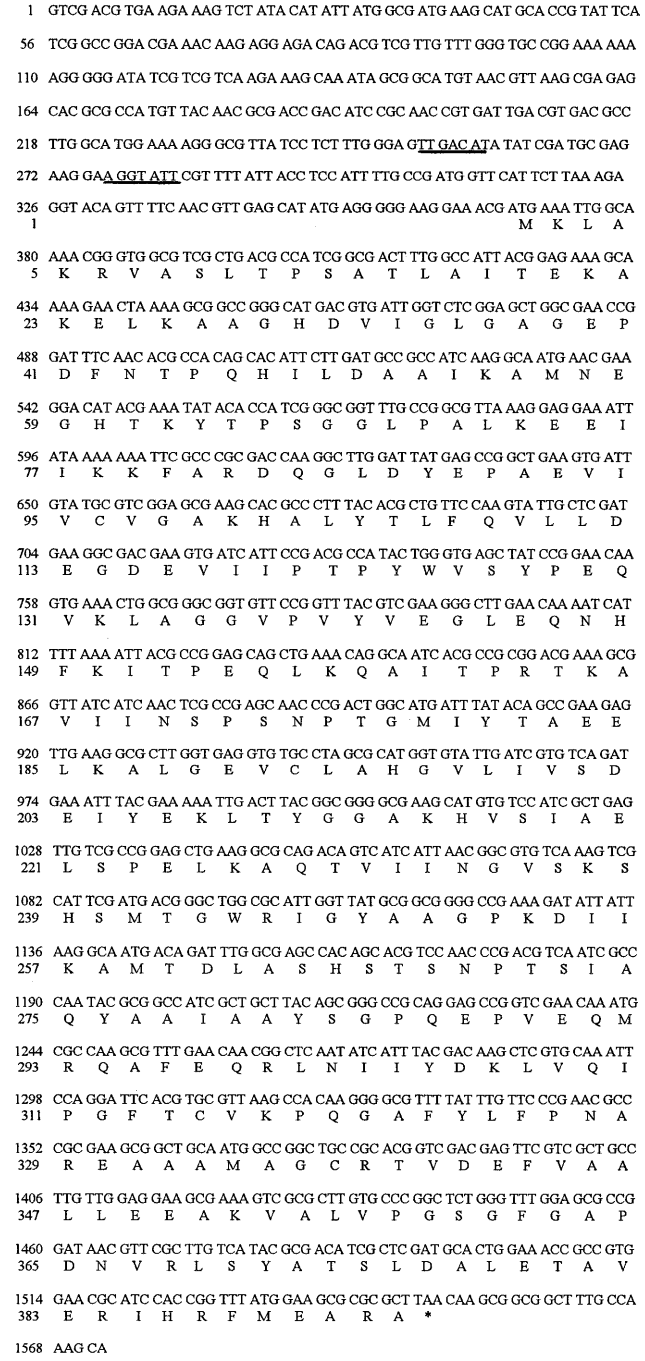

FIG. 2. Nucleotide sequence of the *aspC* structural gene from *B. stearothermophilus* and the putative promoter region upstream of the 5′ end. Potential promoter motifs are underlined. The DNA sequence is numbered beginning at the *Sal*I site outside the open reading frame. The deduced amino acid sequence of the GOT protein is given below the nucleotide sequence and is numbered from the N terminus. *, termination codon.

*Hin*dIII fragment into plasmid pTD1 downstream and in the same orientation as the *gabT* gene (plasmid pKOM1 [Table 1]). In this construct both transaminase genes are transcribed in tandem orientation from the *lac* promoter of the vector. In addition the plasmids pGT2 and pASP1 were cotransformed into *E. coli* W3110. Transformants with a Cm^r Ap^r phenotype were isolated, carrying both transaminase genes on two different expression plasmids. As control strains for the expression studies, transformants of *E. coli* W3110 were used, harboring either plasmid pTD1 or pASP1. The expression strains were grown in Luria-Bertani medium and induced with IPTG as described in Materials and Methods. The specific, thermostable GOT activities in crude extracts from these bacteria were measured as the formation of glutamate from 2-ketoglutarate and aspartate at 70°C. The GOT overexpression in transformants harboring the recombinant *aspC* gene was ca. 10- to 20-fold compared with the untransformed control. The highest overexpression level was observed in the strain overproducing solely the GOT protein (carrying plasmid pASP1) (1,155 nkat/mg of protein), while in the double transformants (carrying plasmid pKOM1 [519 nkat/mg] or pGT2 and pASP1 [418 nkat/mg]) the enzyme production was reduced roughly by half. Similar results were obtained for the specific phosphinothricin transaminase activity in the same overexpression strains (data not shown).

Stereospecific production of L-phosphinothricin in a coupled transaminase process. Both enzymes were partially purified from the overexpression strains *E. coli* W3110::pTD2 (phosphinothricin transaminase) and *E. coli* W3110::pASP1 (GOT). The proteins were concentrated from crude extracts of the bacteria by ammonium sulfate precipitation and further purified by heat precipitation and fractionation on Q-Sepharose HP (see Materials and Methods). The enzymes were immobilized separately to the polymer carrier VA-Epoxy Biosynth (Riedel de Haen) as described in Materials and Methods. Approximately 97% of the phosphinothricin transaminase protein bound to the matrix, exhibiting ca. 26% of the initial catalytic activity. The corresponding values for the GOT protein were 91 and 89%.

The coupled transaminase reaction was performed in batch assays in a total volume of 1 ml at 50°C with the carrier fixed enzymes (43 nkat of phosphinothricin transaminase and 27 nkat of GOT) and a reaction mix containing 500 mM PPO, 100 mM glutamate, and 600 mM aspartate (pH 8.0). After an incubation time of 24 h the catalysts were separated from the product solution by centrifugation. The amino acids in the supernatant were quantified with a Biotronic LC 5001 amino acid analyzer, and the remaining PPO was determined by ^{35}P nuclear magnetic resonance analysis as described in Materials and Methods. The results of one typical reaction were as follows (micromolar concentrations): L-phosphinothricin, 413.5; glutamic acid, 86.0; aspartic acid, 93.5; alanine, 62.5; PPO, 55.0. Under the given conditions, up to 85% of the applied PPO could be converted to L-phosphinothricin, while ca. 10% of unreacted PPO remained in the product solution. The L-phosphinothricin was purified from the reaction broth as described by Schulz et al. (11). The optical purity of the product was >99%.

In all conversions a significant formation of alanine (ca. 10% compared with the applied amino donors) was detected. This reaction is caused by a side activity of the L-phosphinothricin-specific aminotransferase, transaminating the by-product pyruvate to alanine with glutamate as the amino donor (unpublished results), and is most likely the major reason for the incomplete conversion of the substrate PPO to L-phosphinothricin.

DISCUSSION

In the present work we have developed a two-enzyme process for the production of L-phosphinothricin applying aspartate transaminase (GOT) from *B. stearothermophilus* together with the L-phosphinothricin-specific 4-aminobutyrate:2-keto-

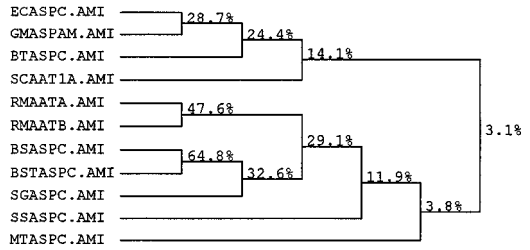

FIG. 3. Sequence similarity diagram of aspartate transaminases from the EMBL database. Amino acid identity scores are indicated. EC, *E. coli*; GM, *Glycine max*; BT, *Bos taurus*; SC, *Saccharomyces cerevisiae*; RM, *R. meliloti*; BS, *Bacillus* sp. strain YM-2; BST, *B. stearothermophilus*; SG, *Streptomyces griseus* (partial sequence); SS, *Sulfolobus solfataricus*; MT, *M. thermoformicicum*.

glutarate transaminase from *E. coli*. We also describe the isolation and characterization of the GOT protein and the cloning and overexpression of the corresponding gene, *aspC*.

The GOT sequence from another thermophilic *Bacillus* species has previously been published by Sung et al. (13). The high level of sequence similarity between the two aspartate transaminases indicates a very close relationship between the two bacterial species. Indeed, Sung et al. (12) have identified *Bacillus* sp. strain YM-2 as a new *Bacillus* species with biochemical and physiological properties falling in between those of *B. sphaericus* and those of *B. stearothermophilus*. In addition, the authors compared the sequence of the thermostable, bacterial GOT with all other GOT sequences in the protein database (13). Interestingly, the highest similarity was observed to the aspartate transaminase from the thermophilic archaebacterium *Sulfolobus solfataricus* (5) (amino acid identity, 34%), while the similarity to the *E. coli* GOT protein, the only other eubacterial aspartate transaminase sequence available at that time, was very low. In the meantime the GOT sequences from the eubacteria *Rhizobium meliloti* (1, 9) and *Streptomyces griseus* (partial sequence) (6) and from the archaebacterium *Methanobacterium thermoformicicum* (15) have been published. Computer analysis of archaebacterial, eubacterial, and eukaryotic GOT sequences (Fig. 3) revealed the existence of two distinct homology groups, one group containing the eukaryotic and the *E. coli* sequences and the other group containing all other known eubacterial sequences. The known archaebacterial enzymes fall also into this second group, even though the degree of sequence similarity is less pronounced.

The high degree of homology between the two thermostable *Bacillus* GOTs is also reflected by the biochemical characteristics of the two purified proteins. Both enzymes show a higher affinity towards the substrate analog cysteine sulfonic acid than to the natural substrate aspartic acid, and in both cases a weak but significant substrate specificity is observed with the sulfur amino acids cysteine and methionine (14). However, there remain also some slight differences in substrate utilization between the two enzymes. The *B. stearothermophilus* aspartate transaminase exhibited a remarkable affinity towards the aromatic amino acids tryptophan, tyrosine, and phenylalanine as amino donors (ca. 10, 2, and 1% compared with the standard substrate aspartic acid), while the GOT from *Bacillus* sp. strain YM-2 was active only with tryptophan to a very low extent (0.1%). In addition, pyruvate could serve as an amino group acceptor only for the *B. stearothermophilus* enzyme. It has to be pointed out that these differences might be in part due to the different reaction temperatures (70°C for the *B. stearothermophilus* enzyme and 50°C for GOT from *Bacillus* sp. strain YM-2) used in the enzyme assays. The enzyme from *B. stearothermophilus* exhibited a more alkaline pH optimum (8.0) and a higher temperature optimum (95°C) than the protein from *Bacillus* sp. strain YM-2 (pH 7.2 and 75°C).

The GOT protein from *B. stearothermophilus* was stable in aqueous solution at 50°C over a period of at least 7 days (data not shown). In contrast to these results the phosphinothricin-specific transaminase from *E. coli* lost its activity in free form after 48 h at 37°C and was suitable only as a biocatalyst in a carrier fixed state (11). Therefore, we decided to apply both enzymes in immobilized form for the L-phosphinothricin production experiments. The immobilization step was more damaging to the phosphinothricin transaminase, which lost 3/4 of its initial catalytic activity during the immobilization, whereas GOT retained 90% of its initial activity.

The coupled two-enzyme process for the production of L-phosphinothricin was very effective. With substrate solutions containing ca. 10% (wt/vol) PPO, conversion rates (PPO→L-phosphinothricin) of up to 85% were achieved. The concentration of amino acid by-products in the reaction broth was only about 3% (wt/vol). These results are major improvements compared with the previously described one-enzyme process (11), where the conversion rates dropped to 76% at similar substrate concentrations and the glutamate concentration in the reaction broth was ca. 24%, causing severe purification problems of the product L-phosphinothricin. Thus, the new process will facilitate product purification significantly. The conversion rates can probably be increased further by eliminating the alanine transaminase side activity present in the enzyme preparations.

ACKNOWLEDGMENTS

We thank K. Tanizawa (Osaka University) for kind provision of the *aspC* clone from *Bacillus* sp. strain YM-2 and D. Tripier (Hoechst AG) for determination of the N-terminal amino acid sequence of the GOT protein from *B. stearothermophilus*.

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