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SPECIFIC IMMUNO-DETECTION OF BENZOATE-PARA-HYDROXYLASE WITH ANTIBODIES RAISED AGAINST SYNTHETIC PEPTIDES

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As a model system for the industrial use of fungal cells in the enzymatic conversion of chemicals, the parahydroxylation of benzoate was studied. To increase the amount of benzoate-para-hydroxylase (BPH, EC 1.14.13.12.) in the cell the gene coding for the enzyme (*bphA*) was cloned and expressed in *Aspergillus niger*. Detection of the enzymatic activity of the protein was not reproducible. It was decided to raise an antiserum for immuno-detection purposes. Sufficient benzoate-para-hydroxylase for immunization could not be obtained; therefore the synthetic-peptide strategy was used. We demonstrate that synthesis of antigenic determinants, can be useful in the production of highly specific reagents for the detection of proteins. The availability of monospecific polyclonal sera opens new possibilities in functional studies and purification of benzoate-para-hydroxylase. © 1990 Academic Press, Inc.

Already in 1971 a synthetic peptide corresponding to a part of the tobacco mosaic virus coat protein was used for raising antibodies against the native protein [1]. Since then the use of peptides has become an important tool in the production of specific antibodies. We have shown that short synthetic peptides, predicted from the DNA base sequence, can induce antibodies which react with the peptides and also with the native proteins from which they are derived [2]. In some cases the use of synthetic peptides is even preferable to intact native protein, especially when the protein belongs to a group of homologous proteins where the presence of immunodominant class specific structures could prevent the development of subclass specific antibodies [3,4]. Synthetic peptides might also become important in the production of vaccines [5]. Furthermore applications are found in those cases in which only the DNA sequence of the protein is known [2] and when the protein can not be purified in sufficient quantities to be used for immunization, as was demonstrated in the current study.

It is assumed that proteins contain a limited number of antigenic determinants and that only peptides corresponding to those determinants are suitable for production of anti-native protein antisera, provided that the peptides have a similar conformation as the sequences in the protein.

Abbreviations: *A. niger*, *Aspergillus niger*; BPH, benzoate-para-hydroxylase; KLH, keyhole limpet haemocyanin; MBS, *m*-maleimido-benzoyl-*N*-hydroxysuccinimide-ester; BSA, bovine sera albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

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Since neither the antigenic determinants nor the three-dimensional structure of the BPH protein were known the sequences for synthesis where chosen on theoretical considerations alone. To improve the immunogenicity of the selected short sequences, the peptides were linked to an immunogenic carrier protein.

In this report we describe the selection of the BPH sequences for peptide synthesis, the methods to couple the peptides to the antigenic carrier molecules and the capacities of the conjugates to elicit anti-peptide antibodies, against the peptides, which crossreact with the native protein. We demonstrate that this approach is useful to raise mono-specific polyclonal antibodies which can be used for immuno-detection of the enzyme.

Materials and Methods

Peptide synthesis and conjugation. Selection of the peptides was based on prediction-plot patterns including the hydrophilicity, surface probability, chain flexibility and secondary structure Together they give rise to the antigenic index [6,7]. All peptides were extended with an extra cysteine at the carboxyl terminus to facilitate chemical coupling to the carrier protein. Coupling of the peptides to keyhole limpet haemocyanin (KLH) with *m*-maleimido-benzoyl-*N*-hydroxysuccinimide-ester (MBS) was carried out according to the method described by Kitagawa et al., [8] with some modifications [2]. Coupling to bovine sera albumin (BSA) with the use of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was performed as described by Boersma et al., [9]. The peptides were synthesized, according to the amino-acid sequence deduced from the nucleotide sequence of the *A. niger bphA* gene [Van Gorcom, et al., unpublished results]. The synthesis was performed on polystyrene resin (1% crosslinking) with tertiary-butyl-oxycarbonyl amino-acids, following the solid phase method as described by Merrifield [10]. After hydrogen fluoride cleavage [11] of the completed peptide the amino acid compositions were determined by HPLC analysis according to the method of Janssen et al. [12].

Anti-peptide antisera. Female rabbits were immunized intradermally three times with the conjugate with a monthly interval. The initial injections administered 250 μg peptide-carrier conjugate emulsified in Freund's complete adjuvant. The conjugate for the second booster injection was emulsified in Freund's incomplete adjuvant. Fourteen days after the second booster injection the sera were collected by bleeding of the ear vein. The sera were screened for anti-peptide reactivity by a direct and an inhibition enzyme-linked-immunosorbent assay (ELISA).

Direct and inhibition ELISA. The ELISA was carried out as previously described by Boersma et al. [13]. In the inhibition assays, which were performed at the same conditions as the direct ELISA, the peptide-induced antisera dilutions and control sera were preincubated for one hour at 25°C with antigen before they were added to the wells.

Western blot analysis. Western blot analysis with the peptide antisera was performed on extracts of mycelia of two *Aspergillus niger* transformants, both having multiple copies of the *bphA* gene. The assay was carried out according to a standard procedure [14] with fractions of cell-extracts of benzoate induced and non induced *Aspergillus niger* strains.

Results

Peptide selection and amino acid composition. Depicted in figure 1 is the graphical representation of the data concerning the hydrophilicity-, surface probability-, flexibility-, antigenic index- and secondary structure predictions of the BPH protein. Examination of those data, revealed three sequences with a consecutive congenial (positive) antigenic index. These peptides [Table 1] were synthesized with an additional cysteine at the carboxyl terminus to enable coupling to the carrier-protein. The amino acid composition of the peptides was confirmed by acid

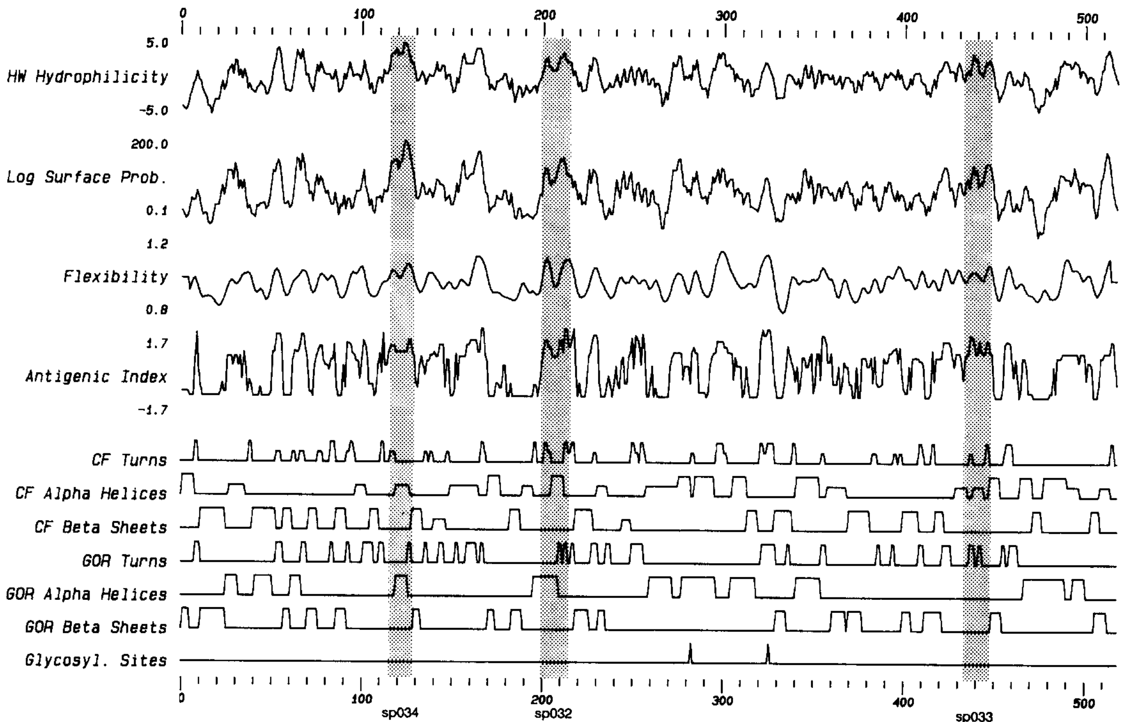

Figure 1.
A graphical representation of the structure prediction data for the aminoacid sequence of benzoate-para-hydroxylase. The three selected sequences with a consecutive high antigen index are depicted in the shaded areas.

hydrolysis followed by HPLC analysis. Purity of peptides SP032, SP034 and SP033 exceeded 95%, 95% and 75% respectively. In contrast to peptides SP032 and SP034, peptide SP033 did not dissolve in 5% acetic acid and no optimal conjugates were obtained.

Direct and inhibition ELISA. For each synthetic peptide the production of anti-peptide antibodies after three immunizations is demonstrated in figure 2. The KLH-MBS conjugates of all the three peptides induced antibodies which not only specifically recognized the respective homologous (non-conjugated) peptides but in addition reacted also with these peptides conjugated

TABLE 1
AMINO-ACID SEQUENCES OF THE SYNTHETIC PEPTIDES AND THEIR RESIDUE POSITION WITHIN THE BPH MOLECULE

peptide code	sequence	residue no
SP032	NH2-Leu-Asp-Lys-Gly-Lys-Asp-Phe-Ala-Glu-Met-Arg-Lys-Thr-Pro-Asp-Ser-Pro-*Cys*-COOH	199-215
SP033	NH2-Phe-Val-Pro-Glu-Arg-Trp-Asp-Pro-Ala-Arg-Leu-Thr-Pro-Arg-Gln-Lys-*Cys*-COOH	433-448
SP034	NH2-Asn-Thr-Arg-Asp-Arg-Ala-Glu-His-Thr-Arg-Lys-Arg-Lys-Thr-Val-*Cys*-COOH	115-129

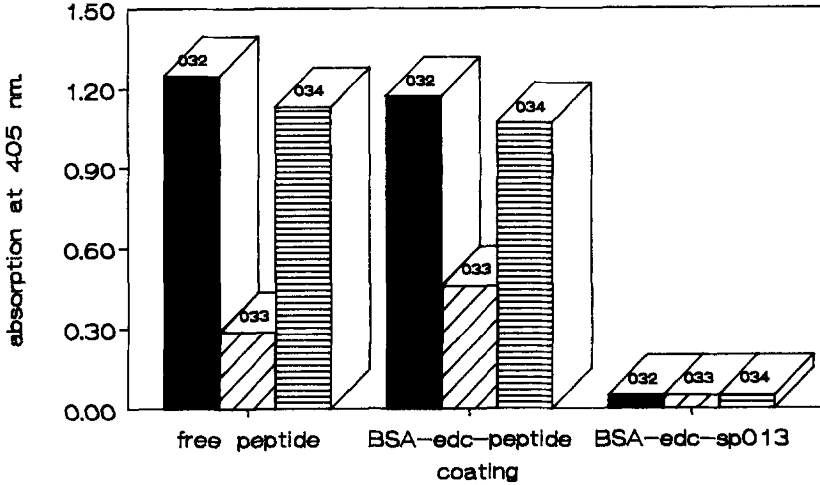

Figure 2.
The antibodies in the serum of rabbits immunized with KLH-MBS-SP032 (black area's), KLH-MBS-SP033 (crossed area's) and KLH-MBS-SP034 (hatched area's) were tested in a direct ELISA on a coating of the peptides the peptide conjugates and an irrelevant peptide conjugate (BSA-EDC-SP013). The results of 1:50 sera dilutions are shown.

with an alternative coupling method (EDC) to another carrier (BSA). No response was measured when the sera were tested on a coating of an irrelevant peptide conjugate (BSA-EDC-SP013). The highest response was observed with peptides SP032 and SP034. In the inhibition assays it was shown that the anti-peptide antiserum response could be inhibited with the specific peptides or specific peptide conjugates. Preincubation with peptides and peptide-conjugates not related to BPH did not inhibit the anti-peptide response et al. [Fig. 3]. Similar results were obtained with inhibition assays with the anti-peptide SP033 and SP034 sera respectively (results not shown).

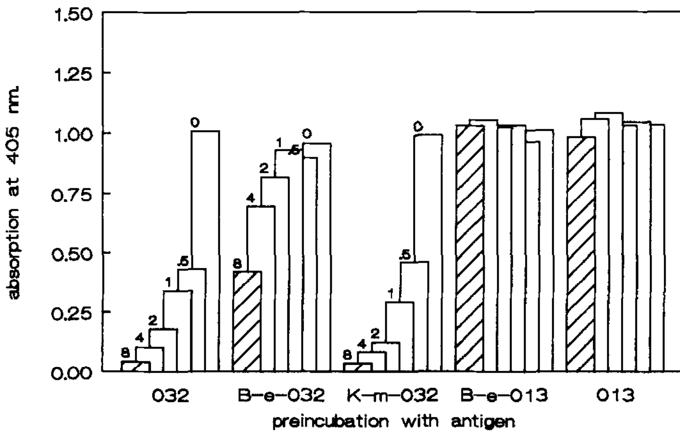

Figure 3.
Serum of a KLH-MBS-SP032 immunized rabbit, after 1 hour preincubation with increasing amounts of specific antigens SP032, BSA-EDC-SP032, KLH-MBS-SP032 or irrelevant antigens BSA-EDC-SP013 and SP013, was tested in an inhibition assay on directly coated SP032. Indicated is the amount of potential inhibitor added. The results of 1:50 sera dilutions are shown.

strain: T16 T21 T16 T21
induced: - - + +
marker: RM
fraction: P0 P1 S1 P0 P1 S1 P0 P1 S1 P0 P1 S1

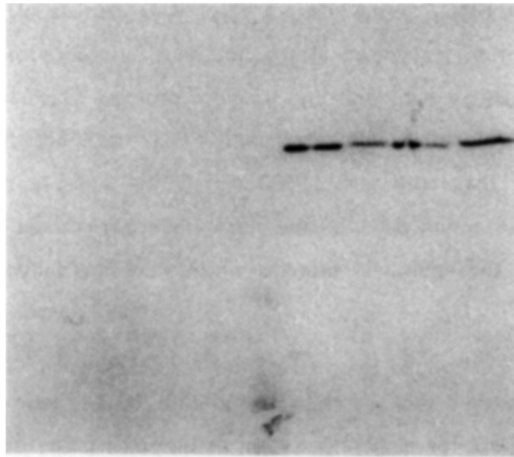

Figure 4.
Comparison of the reactivity of the anti-SP032 antiserum on mycelia extracts (P0=total fraction, P1=membrane fraction, S1=soluble protein fraction) of benzoate induced strains with the non induced strains of transformed *A. niger* in a Western blot. The middle lane contains a molecular weight marker (RM=rainbow marker).

strain: T16 T21 T16 T21
induced: - - + +
marker: RM
fraction: P0 P1 S1 P0 P1 S1 P0 P1 S1 P0 P1 S1

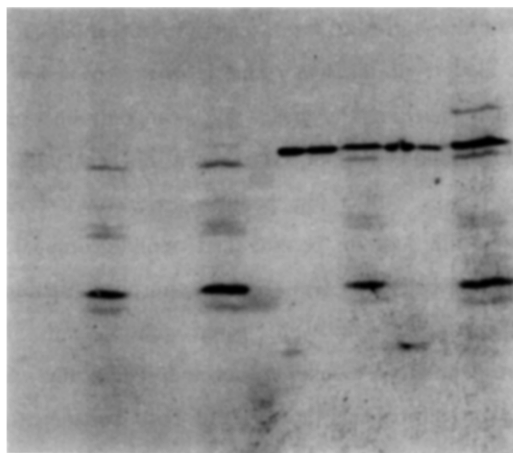

Figure 5.
Comparison of the reactivity of the anti-SP034 antisera on mycelia extracts (P0=total fraction, P1=membrane fraction, S1=soluble protein fraction) of benzoate induced strains with the non induced strains of transformed *A. niger* in a Western blot. The middle lane contains a molecular weight marker (RM=rainbow marker). Double staining was performed with anti-SP032 and SP034 antisera respectively.

Western blot analysis. The total extract (P0), the membrane fraction (P1) and the soluble fraction (S1) of extracts of mycelia of both strains induced to expression of BPH with benzoate reacted very specific with the anti-SP032 sera; only one band (mw=46 kd) was observed on the Western blot. The anti-SP032 serum did not react with extracts of the non-induced strains [Fig. 4]. The anti-SP034 serum recognized also a benzoate induced protein but in addition the anti-SP034 sera showed crossreactivity with one or more proteins which were not induced by benzoate (results not shown). A double staining with anti SP032- and anti SP034 serum respectively was performed to confirm that both anti SP032 and SP034 antisera recognized the same induced protein. The anti-SP034 serum detected the same (induced) protein band as was detected by the anti-SP032 serum and the additional proteins which were not induced by benzoate [Fig. 5].

Discussion

This report describes the successful application of synthetic peptides to generate mono-specific polyclonal antisera for immunodetection of benzoate-para-hydroxylase. With only the DNA sequence as starting point, this approach allows specific detection of BPH, circumventing the difficulties concerning the isolation and purification of the native molecule.

This study demonstrates properties of antibodies raised against three peptides which were derived from separate regions of the benzoate-para-hydroxylase. The antisera raised with conjugates of peptides SP032, SP033 and SP034 showed in ELISA specific responses on their respective peptides and peptide-conjugates. The ability of specific peptides and peptide conjugates to inhibit their respective anti-peptide responses confirmed the above mentioned specificity.

The level of the anti-KLH response of the KLH-MBS-SP033 antisera in ELISA was comparable with the anti-KLH response of the other two peptide antisera. Therefore, the low anti-peptide response of the anti-SP033 sera can not be explained by an inefficient immunization procedure. It is more likely that the low response is due to a low peptide/carrier ratio. Peptide-carrier conjugates, with a high peptide/carrier ratio, can only be obtained if the peptide is soluble during the conjugate formation. Peptide SP033 was not easily dissolvable and therefore it was expected that only conjugates with a low peptide/carrier ratio were formed.

After screening of the sera on peptide and peptide-conjugate, the peptide-specific antisera were used for detection of native protein. For this purpose fractions of extracts of two different transformed strains of *Aspergillus niger* were tested with the anti SP032 and SP034 antisera in a Western blot. The anti-peptide SP032 serum reacted specifically with one protein band (mw=46 kd) in blots of all the mycelia extract fractions of both benzoate induced strains. The molecular weight of the benzoate induced protein was not equal to the expected molecular weight of 58 kd deduced from the DNA sequence analysis [Van Gorcom et al., unpublished results], but integral membrane proteins often show a aberrant behaviour on SDS-polyacryl amide gels. The results also showed that the bulk of the BPH was associated with the membrane fraction. This might explain the difficulties met in attempts to purify the BPH protein from mycelia. The anti-

SP032 sera did not eact with control extract samples of the non-induced strains. Single staining with anti-SP032 and double staining of the Western blot with anti-SP032 and anti-SP034 sera respectively shows that both antisera recognize the same induced protein. However the anti-SP034 antisera recognize not only the BPH protein in the induced-strain fractions but additional proteins as well. These proteins are also present in the non induced strains. Possibly, there is a sequence- or conformational similarity between these proteins and one of the possible conformations represented by the synthesized peptide of the 115-129 amino acid sequence of BPH. Isolation and sequencing of these non-identified proteins could confirm this hypothesis.

This study shows that prediction and synthesis of putative antigenic determinants using only DNA sequences can be a useful tool to the practical biochemist in the production of highly specific reagents for detection of proteins and/or protein fragments. The availability of selected synthetic peptides and corresponding mono-specific polyclonal sera specific for the native sequence opens new possibilities in functional studies and purification of the BPH enzyme.

Acknowledgments

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