

# Performance evaluation of hyperbranched aramids as potential supports for protein immobilization

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Received 28 October 1998; received in revised form 17 March 1999; accepted 30 March 1999

## Abstract

Several adducts of  $\alpha$ -amylase and hyperbranched aramids have been evaluated in terms of their bioactivity performance. Twelve samples of hyperbranched aromatic polyamides, originated from either two  $AB_2$ -type monomers or from five systems formed by reactant pairs ( $A_2 + B_3$  or  $A_3 + B_3$  or  $A_2 + B_4$ ) have been synthesized under different reaction conditions and used as protein supports. Through the addition of a suitable coupling agent, the enzyme fixation step has been carried out by joining the carboxylic groups on or near the outer surface of the aramids to the amino groups of the aminoacids present in  $\alpha$ -amylase.

A rather high efficiency of protein immobilization is observed in our aramids, their binding capability being almost an order of magnitude higher as compared to the commonly used insoluble supports. Each preparation of the immobilized enzyme is analyzed in terms of bioactivity retention as a function of time, as well as stability under various experimental conditions. Enzymatic activity has been evaluated both as  $k_m$  (a measure of the enzyme affinity for the substrate) and as  $k_{cat}$  (used for the determination of catalytic efficiency). Our data show that the linking of the enzyme to the polymeric support leads to the production of three different types of adducts with distinct enzymatic patterns. On the basis of our results it may be inferred that hyperbranched aramids are suitable supports for protein immobilization. The availability of these polymeric structures by simple synthetic preparations can open new perspectives for the development of finely tuned enzyme-based derivatives with pre-defined binding affinity, catalytic capability and structural stability. © 2000 Elsevier Science Ltd. All rights reserved.

**Keywords:** Hyperbranched aramids;  $\alpha$ -Amylase fixation; Enzymatic activity

## 1. Introduction

So far, only a few syntheses of dendrimeric or hyperbranched (HB) aramids have been described in the literature [1–3], presumably because of some experimental drawbacks. However, at least for HB aramids, the synthetic difficulties can easily be overcome by adopting the direct polyamidation reaction, i.e. the one-step reaction between aromatic carboxylic groups, activated by triphenylphosphite and pyridine, and aromatic amino groups.

On these grounds, the synthetic pathways used by our research group in the preparation of seven different structures constituted of HB aramids, together with some properties of the resultant materials, have recently been described [4–6]. The HB aramids have been synthesized either from an  $AB_2$ -type monomer [4,6] or from two-reactant systems

( $A_2 + B_3$  and  $A_3 + B_3$  [4,5];  $A_2 + B_4$  [6]). In the latter ones, the formation of sol and gel fractions in variable amounts has been clearly pointed out by Flory and related to the extent of reaction by various authors. A more detailed analysis of the formation of polyfunctional nonlinear structures has been reported [5]. Depending on the experimental conditions chosen, the sol/gel ratio has been evaluated as a function of conversion [5,6]. The sol fraction of the resultant aramid shows a structure formally very similar to the HB architecture arising from the polymerization of  $AB_x$  monomers and can easily be isolated from the gel fraction by suitable separation methods. Quite recently, the same approach has been suggested by Kakimoto et al. [7]. As mentioned earlier, for the purposes described in the present article a HB aramid synthesized from an ( $A_2 + B_4$ ) pair (*p*-phenylenediamine + pyromellitic acid) has been considered. The detailed description of its synthesis and characterization will be reported elsewhere [6].

Adopting both synthetic pathways and optimizing purifi-

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cation procedures and reaction parameters, as described in detail [4–6], a wider variety of HB aramid structures is now available, as compared to the classical synthetic route based only on  $AB_x$  monomers, and a more detailed evaluation of structure–property relationships is therefore possible.

Two parameters have been found as the most relevant ones in controlling HB aramid properties:

1. regularity rules for the description of how amide groups are sequenced along each branch;
2. branch length, i.e. distance between consecutive branching points.

The classification of our samples has been carried out on this basis, as shown in Section 2.

A full characterization of our HB aramids has been carried out and described [5] for four different systems based on  $(A_2 + B_3)$  and  $(A_3 + B_3)$  reactant pairs, and for two  $AB_2$  monomers and an  $(A_2 + B_4)$  pair [6]. Solubility data, relative amounts of sol/gel fractions in  $(A_2 + B_3)$ ,  $(A_3 + B_3)$  and  $(A_2 + B_4)$  systems, intrinsic viscosity values, full molecular weight distribution and microstructural information by GPC and MALDI-TOF, respectively,  $^1\text{H}$  and  $^{13}\text{C}$  NMR, UV, IR and Raman spectral data, and thermal behavior by DSC and TGA are given in the above references [4–6]. In the present article, the attention is focused on the capability of our HB aramids to act as rigid supports for a specific enzyme ( $\alpha$ -amylase), covalently bound to them. Rigidity is provided by the aromatic nuclei, *para*- and *meta*-connected through  $-\text{CONH}-$  groups.

The immobilization of proteins is a fundamental step in constructing enzyme-based reactor systems. Polymer molecules are very useful in this respect, as they possess a large number of binding sites allowing high protein loading. Recently, several polymeric supports have been described in the literature, including hydrophilic compounds [8–11] (such as polysaccharides [8,9]), hydrophobic polymers [12–15] (such as polydimethylsiloxane [15]), amphipatic polymers (such as monomethoxy-polyethylene glycol [16]). All these materials have found several applications in various areas, e.g. medical, therapeutic and industrial treatments. However, their flexible nature may be a strong limitation to their performances, when rigidity of the enzyme-supporting structures is required. For instance, in biotechnological processes with high flow rates, support rigidity minimizes shrinkage/swelling phenomena which may cause a relevant loss of bioactivity.

## 2. Experimental

### 2.1. Enzyme fixation

As mentioned in Section 1 detailed description of the procedure followed in the syntheses of monomers and reactants, as well as in the purification of all the components of the polycondensation reaction medium, and in the synthesis

of HB aramids is given elsewhere [4–6]. In particular, full molecular characterization, including data on molecular weights of our HB aramids, will be reported shortly [6]. Only the enzyme immobilization reaction is herein described in detail. The method involves formation of a covalent bond between the carboxylic groups of aramids and the amino groups present in the aminoacid residues of  $\alpha$ -amylase (Novo Nordisk) by means of 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride (EDC—Pierce) as a coupling agent [17]. 20 mg of aramid are incubated with 30 mg of enzyme and EDC ( $10^{-2}$  M) in 0.5 ml total volume for 17 h at room temperature. The reaction is performed in acetate buffer ( $10^{-1}$  M) at pH 4.5. The immobilized  $\alpha$ -amylase is then stored at  $10^\circ\text{C}$ . The amount of enzyme coupled to the polymeric support is evaluated both by a direct protein determination method and by the difference between total protein added to the reaction mixture and free protein. No appreciable difference between the two methods is found. The protein assay on the immobilized enzyme is performed by bicinchoninic acid (BCA Kit from Pierce) following the manufacturer instructions, based on the reducing properties of proteins [18]. Functional properties of the immobilized protein are compared to those of the free enzyme.

### 2.2. Enzymatic assay for $\alpha$ -amylase

The catalytic activity of  $\alpha$ -amylase is evaluated by an enzymatic assay where an insoluble substrate is used. Namely, insoluble starch covalently labeled with the reactive dye Remazol Brilliant Blue (RBB—Fluka) is cleaved by  $\alpha$ -amylase leading to the formation of blue colored malto-dextrins and oligosaccharides that become soluble after the enzymatic treatment [19]. The supernatant hydrolysis products are spectrophotometrically determined at 590 nm.

The enzymatic activity as a function of time is evaluated three to six months after immobilization and expressed as a percentage of maximum activity obtained for each adduct immediately after the coupling reaction.

The effect of pH on the catalytic activity is evaluated under standard conditions and the buffers used are acetate buffer (0.5 M pH 4.7), phosphate buffer (0.5 M pH 6.5) and carbonate buffer (0.5 M pH 9). The activity is indicated as percentage of the maximum activity obtained at pH 6.5.

### 2.3. Analysis of kinetic data

$k_m$  and  $V_{\text{max}}$  are calculated from the Lineweaver–Burk plot.  $k_m$  is expressed in mg/ml, as the starch is a polymolecular substrate that cannot be expressed in molar concentration.  $V_{\text{max}}$  is given as variation of optical density at 590 nm per unit time per mole of enzyme and used for the calculation of  $k_{\text{cat}}$ .

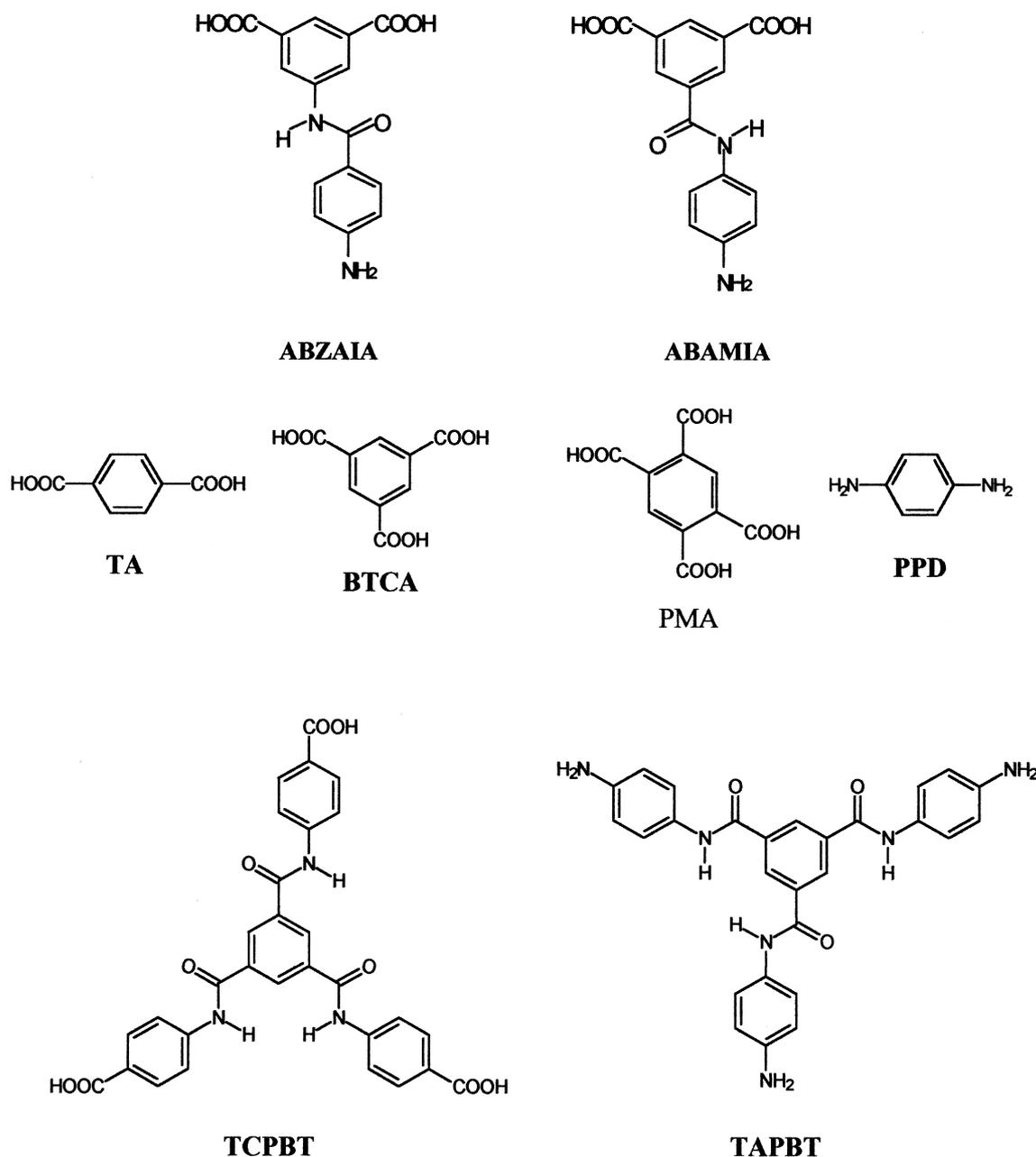


Fig. 1. Monomers and reagents used for the synthesis of HB aramids.

### 3. Results and discussion

#### 3.1. Hyperbranched aramids

Seven different structures of our HB aramids have been originated by the step polycondensation of monomers or reagent pairs represented in Fig. 1 and listed in the chosen combinations in Table 1. In the second column of the above table the sequence of amide groups along each branch is compared to that of poly(*p*-benzamide) (PBA) or that of poly(*p*-phenylene terephthalamide) (PPDT). In PBA, the -CO- and -NH- groups are regularly alternating along the chain. In PPDT, on the contrary, a regular inversion of

the above groups is present in the CRU sequences. 'Disordered' sequences here have the meaning of more complex regularity rules describing the running of -CO- and -NH- groups in the amide sequences along each branch. The net effect of these complex rules means in practice a local 'disorder'.

The notation L(Ar) in the last column of Table 1 gives the number of aromatic rings present within the trifunctional branch points and is a measure of the branch length, higher values corresponding to larger 'holes' in the structure of the specific HB aramid. As indicated in Section 1, the above two parameters can fully describe the structure and the behavior of our HB aramids, and provide useful structure-property

Table 1

Synthesis and microstructural characterization of HB aramids (ABZAIA: 5-(4'-aminobenzoylamino)isophthalic acid; ABAMIA: 5-(4'-aminophenylcarbamoyl) isophthalic acid; PPD: p-phenylenediamine; BTCA: trimesic acid; PMA: pyromellitic acid; TAPBT: 1,3,5-tris(4'-aminophenylcarbamoyl) benzene; TA: terephthalic acid; TCPBT: 1,3,5-tris(4'-carboxyphenylcarbamoyl)benzene; L(Ar) is the number of aromatic rings present within the trifunctional branch points)

Monomer or reagent pair	Sequence analogy of amide groups	L(Ar)
ABZAIA	PBA	1
ABAMIA	PPDT	1
PPD + BTCA	PPDT	1
PPD + PMA	PPDT	1
TAPBT + TA	PPDT	3
PPD + TCPBT	Disordered PPDT	3
TAPBT + TCPBT	Disordered PPDT	2

relationships [5,6]. For instance,  $T_g$  values are linked to the degree of order followed by the –CO– and –NH– groups running along the branches [5]. For a typical ABZAIA—derived polymer  $M_w$ , determined by Multi Angle Laser Light Scattering (MALS) [6], is ca.  $1.1 \times 10^5$ . Similar values are expected for the other HB aramids.

Rigid supports made of HB polymeric materials, as compared to the more flexible ones, are potentially very useful for biomolecule fixation. As mentioned in Section 1, rigidity of the support would minimize shrinkage and swelling effects, thus preserving the activity of the fixed biomolecule. Twelve different adducts of  $\alpha$ -amylase and HB aramids have been prepared on the basis of the seven structures arising from the reacting systems listed in Table 1. Differences among them can easily be explained inasmuch as aramids derived from the same chemical source may differ from one another on the basis of chosen reaction

parameters (e.g. composition of the reaction medium, polyamidation time, content of sol fraction) and therefore lead to different adducts.  $\alpha$ -Amylase has been chosen as reference biomolecule to test the linking capability and bioactivity retention of our HB adducts.

### 3.2. Enzyme immobilization and protein content

As shown in Table 2 (column 4), the level of enzyme immobilization (in terms of enzyme/aramid w/w ratio) is very high and rather comparable (between 70 and 86%) for nine of the 12 HB aramids. These data emphasize the high reactivity of our materials, able to covalently link the specific enzyme with efficiency almost an order of magnitude higher than the most common insoluble supports. As evidenced by the earlier data, efficiency differences among the various HB aramids are minor, whereas the simple enzyme adsorption onto the polymeric substrate gives a much lower amount of immobilized protein (<10%). In addition, the latter procedure causes a relevant leaking of biocatalyst from the support in a rather short time.

### 3.3. Evaluation of enzyme activity

Catalytic properties of the immobilized enzyme have been evaluated for the twelve adducts prepared as described earlier. As shown in Table 2 (columns 5–7) a full kinetic analysis has been performed and the measure of  $k_m$ ,  $V_{max}$  and  $k_{cat}$  allowed grouping of the adducts in three different sets, each characterized by a rather homogeneous enzymatic behavior.

In the bottom group (six adducts), a high affinity of the enzyme for its substrate (represented by low  $k_m$  values) together with a low  $k_{cat}$  is observed, while in the top group (AB2, Y1 and Y8) a lower affinity (high  $k_m$  values) is

Table 2

$\alpha$ -Amylase linked to the hyperbranched aramids and kinetic data of its bioactivity

Sample code	Reagent pair or monomer	L(Ar)	Immobilized enzyme/aramid (w/w) <sup>a</sup>	$k_m$ (mg/ml) <sup>b</sup>	$V_{max}$ ( $\Delta A s^{-1}$ ) <sup>a</sup>	$10^{-3} k_{cat}$ ( $s^{-1}$ ) <sup>a</sup>
AB2	PPD + PMA	1	0.80	19	0.18	85.1
Y1	PPD + BTCA	1	0.70	16	0.07	49.3
Y8	PPD + BTCA	1	0.82	26	0.10	39.2
AB6	ABAMIA	1	0.72	6	0.02	11.4
V17	ABZAIA	1	0.72	8	0.03	18.9
V14	ABZAIA	1	0.62	13	0.03	10.0
V13	ABZAIA	1	0.86	3	0.01	5.1
Y22	ABZAIA	1	0.81	4	0.02	7.5
V15	ABZAIA	1	0.70	5	0.01	5.1
Y34	TAPBT + TCPBT	2	0.58	2	0.01	4.7
Y33	TAPBT + TA	3	0.60	4	0.02	14.9
AB50	PPD + TCPBT	3	0.78	2	0.01	3.5
	<b><math>\alpha</math>-amylase(free enzyme)</b>			<b>6</b>	<b>0.41</b>	<b>140.4</b>

<sup>a</sup> Average of two determinations (standard deviation does not exceed 15%).

<sup>b</sup> Michaelis–Menten constant is expressed as starch-RBB concentration (mg/ml).

Table 3

Residual enzymatic activity (%), (average of two determinations (standard deviation does not exceed 15%)) as a function of storage time and pH (measured seven days after the crosslinking reaction)

Sample code	Three months	Six months	pH 4.7	pH 6.5	pH 9
AB2	100	100	28.8	100	13.2
Y1	100	100	54.5	100	40.9
Y8	70	54	–	–	–
AB6	90	81	48.5	100	34.3
V17	100	100	–	–	–
V14	85	78	–	–	–
V13	50	37	–	–	–
Y22	100	100	30	100	15.6
V15	100	100	–	–	–
Y34	100	100	40	100	33.6
Y33	–	–	86.7	100	63.3
AB50	100	74	–	–	–
<b>Free amylase</b>	100	100	50.6	100	21.0

associated with a much higher catalytic activity; in the third group, intermediate kinetic behavior has been observed. As indicated earlier, the lowering of  $k_m$  values emphasizes a higher affinity between the catalytic site of the enzyme and the substrate, while an increase of  $k_{cat}$  is indicative of a higher turnover number, i.e. a higher amount of product obtained per unit time.

In general, samples characterized by a distance between the trifunctional branching points equal to 3 or 2 L(Ar) show a greater affinity for the substrate than those characterized by 1 L(Ar).  $k_{cat}$  values have a distribution that is just the opposite: it is clear therefore that a less compact structure makes the interaction between the active site of the enzyme and the substrate easier, but does not provide a stronger enzymatic activity. On the contrary, in the more compact structure 1 L(Ar), a lower substrate affinity is associated to a higher catalytic efficiency. Besides these structural effects, more subtle differences lie on the specific preparation procedure of our HB aramids, which may lead to additional structural variations, such as those induced by different degrees of branching developed during the synthesis.

### 3.4. Stability and effect of pH on the catalytic activity of the immobilized $\alpha$ -amylase

The stability of the immobilized enzyme has been evaluated and, as shown in Table 3, a high bioactivity retention is observed in almost all the samples analyzed three and six months after the binding reaction. This result, partially linked to the structural and biochemical properties of native  $\alpha$ -amylase, emphasizes the positive role of immobilization which, at least, does not reduce the natural stability of the enzyme.

The pH dependence of the activity of immobilized  $\alpha$ -amylase has been compared to that of the unmodified enzyme. In the 4.7–9.0 range the optimum pH is 6.5 in all cases; at pH 4.7 and 9.0, a decrease of the enzymatic activity is observed for both the immobilized and the free enzyme;

however, at pH 9.0 the residual activity of the immobilized enzyme in most samples is significantly higher than that of the unmodified form.

Thus, the immobilization process provides a structural stability, preventing an irreversible unfolding of the enzymatic protein. This effect is not specific for proteins immobilized on rigid aromatic polymers, as it has already been described for enzymes coupled to a variety of insoluble supports [20].

## 4. Conclusions

Hyperbranched aramids are suitable supports for enzyme immobilization. The adduct formation process leads to three different groups of adducts, with adjustable biochemical and catalytic properties. Furthermore, a structural stability of the immobilized enzyme at different pH has been observed. The availability of these polymeric structures could open new perspectives for the development of finely tuned enzymatic adducts and outline strategies for obtaining enzyme derivatives with pre-defined binding affinity, as well as catalytic and structural stability.

Molecular modeling of the various HB structures could be very helpful in this respect and able to interpret differences in behavior among the twelve adducts [21].

## Acknowledgements

This research has been partially supported by MURST (Ministero dell'Università e della Ricerca Scientifica) cofinanziamento funds. The experimental work of Alya Boulares, Andrea da Rin and Valentina Canepa in the preparation and characterization of HB aramids has been highly appreciated.

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