ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 9 (2000) 97–105

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Molecular (or bio-) imprinting of bovine serum albumin

Christopher J. Slade *

Food Macromolecular Science Department, Institute of Food Research, Earley Gate, Whiteknights Road, Reading, RG6 6BZ, UK

Received 25 May 1999; accepted 10 August 1999

Abstract

The lyophilization of proteins in the presence of template molecules has been suggested as a means of creating ''de novo'' binding and catalytic sites. The bio-imprinting of bovine serum albumin (BSA) was investigated using the binding of *p*-hydroxybenzoic acid (*p*HBA) and the β-elimination of 4-fluoro-4-(*p*-nitrophenyl)butan-2-one as model systems. It was found that both binding and catalytic activity could be enhanced by a factor of approximately 3 over that of the native protein but that no specificity was introduced. It was also found that activity was restricted to active groups on the surface of the imprinted proteins which could be influenced by this technique. A synergistic effect between lysine and aspartic acid groups was observed. This suggested that ionised carboxylic acid and amine groups are involved in bio-imprinting and further suggests ways in which the technique could be developed to produce novel protein based catalysts.

Keywords: Proteins; Conformational memory; Organic solvents; Molecular imprinting; Bovine serum albumin; Catalysis; Binding

1. Introduction

The use of enzymes in organic synthesis [1] is now well documented and their ability to perform reactions in anhydrous solvents [2] increases their potential further. The insight into the creation of artificial enzymes though came from the statement of Pauling [3] that, ''Enzymes are molecules that are complementary to the structures of the activated complexes of the reactions that they catalyse.'' This concept led to the development of catalytic antibodies which are antibodies that have been raised against a transition state analogue of the reaction under study. They represent a potentially important synthetical tool as they can be used where no natural enzyme exists, although they are expensive to produce.

The reports by Braco et al. [4] and Dabulis and Klibanov [5], which involved the lyophilization of an excess of a template molecule with a protein such as bovine serum albumin (BSA), have claimed the formation of binding sites in the protein that were specific for the original template. When this is considered together with the observations that enzyme activity [6], substrate specificity [7,8] and enantioselectivity [9] can be markedly effected by lyophilising or precipitating an enzyme with a given substrate, it suggests that a new approach to ''synthetic enzymes'' complementary to catalytic antibodies is possible. The constraining point of this technique, now called bio imprinting [10], is

* e-mail: christopher@jslade1.freeserve.co.uk

1381-1177/00/$ - see front matter
PII: S1381-1177(99)00089-2

that the protein or enzyme must remain in an anhydrous or nearly anhydrous solvent during the reactions otherwise the acquired specificity is lost [11]. The application of this concept to BSA is appealing due to its cheapness and abundance, two aspects that are necessary if a protein with template induced catalytic activity is to be commercially viable.

However, as BSA is a complex protein with numerous natural binding and catalytic sites, a thorough understanding of the imprinting process is required to facilitate its application. Fifty years of investigation have shown that the serum albumins can catalyse processes as diverse as the Diels-Alder [12] and eliminations reactions [13]. Also, the serum albumins are well known for their ability to bind many different compounds in a variety of binding sites with binding constants that range from 10^{16} M^{-1} for Cu(II) to 10^{8} M^{-1} for bilirubin to 10^{7} M^{-1} for long chain fatty acid and to 10^{5} M^{-1} for 2-acetyl benzoic acid (aspirin). This makes the addition of a new catalytic process, with the aim of forming a pure product, difficult. The identification of naturally active sites has been aided by the publication of the crystal structures of the common albumins [14]. In addition, certain dyes, such as fluorescein–isothiocyanate (FITC) [15], can be specifically coupled to albumin to interact with various binding and/or catalytic sites.

It was previously suggested that the technique of bio-imprinting of BSA generated specific binding cavities in the protein [4,5]. However, it has not been conclusively shown that the results were due directly to conformational changes. Only a limited set of compounds were studied and other possible explanations were not explored. Also, the non-imprinted BSA used as a control was obtained by lowering the pH of the solution with HCl before lyophilization. This was necessary to simulate the pH achieved by the template molecule because the structure of BSA is dependant upon the pH of the solution it is in. Such controls may be inappropriate as no attempt was made to remove this acid before binding experiments were undertaken. The difference in binding between this non-imprinted control BSA and the imprinted BSAs led to the assumption that specific binding sites had been created by the process. A more detailed understanding of the events that take place during imprinting would increase our knowledge of this phenomena and so assist in developing the necessary protocols if bio-imprinting is to be a feasible method for the introduction of ''de novo'' catalytic sites into proteins.

The study described in this paper attempts to identify the processes and/or changes that take place in BSA when it is lyophilised with a template according to the method described by Braco et al. [4]. Firstly, by investigating the binding of *p*-hydroxybenzoic acid (*p*HBA) in anhydrous acetonitrile to a number of imprinted and non-imprinted BSAs. Then, secondly, the catalytic β-elimination ability of BSA imprinted with a variety of templates, was investigated using 4-fluoro-4-(*p*-nitrophenyl)butan-2-one as substrate Fig. 1(**1**). This substrate has previously been used in studies of catalytic antibodies [16], molecular imprinted polymers [17,18] and bio-imprinted proteins [13,19].

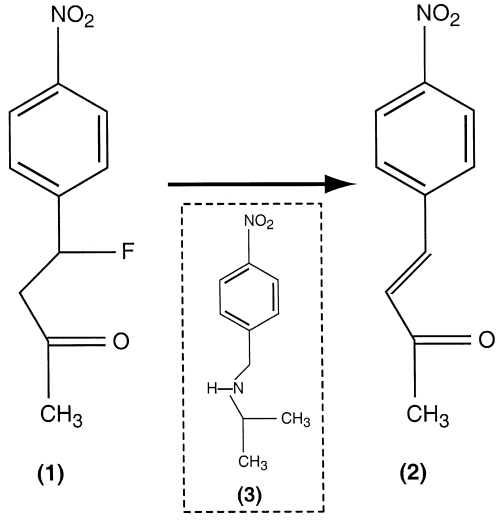

Fig. 1. β-Elimination of 4-fluoro-4-(*p*-nitrophenyl)butane-2-one to 4-(*p*-nitrophenyl)-3-buten-2-one. Also showing the transition state analogue *N*-isopropyl-4-nitrobenzylamine (**3**).

2. Material and methods

Fatty acid-free BSA (cat. A6003), poly-L-lysine hydrobromide (mol. wt. 30 000–70 000), poly-L-aspartic acid sodium salt (mol. wt. 15 000–50 000) and phosphate buffered saline (PBS) (cat. P4417) were obtained from Sigma, Poole Dorset, UK. *O*,*O*′-Bis(2-aminopropyl)-polyethylene glycol 500 (Jeffamine ED-600) was obtained from Fluka Chemicals, Gillingham Dorset, UK. 4-Fluoro-4-(*p*-nitrophenyl)-butan-2-one (**1**) and *N*-isopropyl-4-nitrobenzylamine (**3**) were prepared as described previously [18]. All other chemicals were obtained from Aldrich Chemical, Gillingham, Dorset, UK.

2.1. Blocking of active site lysines in BSA with FITC

Lysine groups on BSA were reacted with FITC as described by Kikuchi et al. [20]. The quantity of fluorescein bound to BSA was determined from the absorbance maximum at 500–501 nm using an extinction coefficient of 75 900 M^{-1} cm^{-1}. Increasing molar quantities of FITC were then used to produce BSAs with larger FITC/BSA ratios.

2.2. Imprinting of proteins

BSA was imprinted, according to the method of Braco et al. [4], with a variety of templates which included: *p*HBA, Jeffamine ED-600 (a high molecular weight base soluble in polar solvents) and *N*-isopropyl-4-nitrobenzylamine. BSA was also freeze dried with varying quantities of hydrochloric acid to give protein preparations at pH 1.9, 2.2, 3.1, 4.0, 5.1 and 6.8. A 1:1 mixture, on a molar basis, of salt free poly-L-lysine and poly-L-aspartic acid was imprinted with only the Jeffamine ED-600 template while both poly-L-lysine and poly-L-aspartic acid were imprinted with *p*HBA. Previous studies [4,5,19] have shown that the washing procedure is adequate to remove all of the free template. A non-imprinted control was prepared in exactly the same way except that the template was omitted. All samples were stored under vacuum.

2.3. Determination of pHBA binding

The *p*HBA binding kinetics of the imprinted and non-imprinted proteins were determined using the following procedure with the *p*HBA concentration being varied from 0.09 to 2.34 mM. An accurately weighed sample of the protein (6.6 mg) was suspended in a solution of *p*HBA in anhydrous acetonitrile (1.0 ml). The samples were sonicated for 5 min to produce a fine dispersion of protein and then placed on an orbital shaker for 16 h at room temperature. Analysis of the resulting *p*HBA concentration in the filtered supernatants, with dilution where necessary, was performed by HPLC on a Gilson analytical system fitted with a LDC 3100X variable wavelength UV detector set at 254 nm and a spherisorb ODS2 (25 cm, 5 μm) column. Elution was achieved at 1.0 ml/min using a linear gradient of water–acetonitrile (60%–90% v/v). To quantify the results, a calibration graph was constructed using accurately weighed samples of *p*HBA and all experiments were performed in at least duplicate.

2.4. Determination of catalytic activity (aqueous reaction)

The catalytic activity in aqueous solution of BSA and the poly amino acids towards β-elimination of 4-fluoro-4-(*p*-nitrophenyl)butan-2-one (**1**) was followed spectrophotometrically at 330 nm on a Perkin Elmer λ-15 spectrophotometer as described previously [13]. The aqueous assay was performed directly in a spectrophotometric cell at 40°C in PBS at a final protein concentration of 1 μM. The reaction was initiated by adding 15 μl of substrate (**1**) to give a final concentration of 750 μM in a volume of 1 ml. The reaction rate was calculated from the observed increase in elimination

product, 4-(*p*-nitrophenyl)-3-buten-2-one (**2**), using the extinction coefficient of 8.3×10^3 M^{-1} cm^{-1} calculated for the change in absorbance at 330 nm [18].

2.5. Determination of catalytic activity of imprinted and non-imprinted proteins

Catalytic activity of imprinted proteins was determined in dry acetonitrile at a protein concentration of 0.1 mM. To an accurately weighed protein sample (either imprinted or non-imprinted) was added an acetonitrile solution (1.0 ml) of substrate (**1**) at either 13, 22, 55 or 114 mM. The samples were sealed and then incubated at 50°C. At 1 h intervals an aliquot (20 μl) was withdrawn, diluted with acetonitrile (1.0 ml) and the absorbance at 330 nm measured. The concentration of product was then calculated as described above.

3. Results and discussion

BSA was freeze dried with a variety of templates and at a range of acid adjusted pH (1.9–6.8) as stated above. These sample were then challenged with various solutions of *p*HBA that ranged in concentration from 0.09 to 2.34 mM. The binding of *p*HBA by each BSA sample was then measured. The BSA imprinted with *p*HBA bound 7.7 mol *p*HBA/mol BSA, which showed a small increase over BSA that was freeze dried in the absence of any template (5.8 mol/mol). This was slightly lower than the binding of BSA imprinted with Jeffamine ED-600 (9.5 mol/mol), a high molecular weight primary amine. More importantly, the dependence of binding upon the pH of the prior lyophilized solution is clearly demonstrated in Fig. 2. This shows that, as the lyophilization pH decreased so did the binding of *p*HBA. The unsuitability of the acid adjusted BSA as a control, as used in the early work, was further demonstrated by redissolving the imprinted BSA and acid adjusted non-imprinted BSA separately

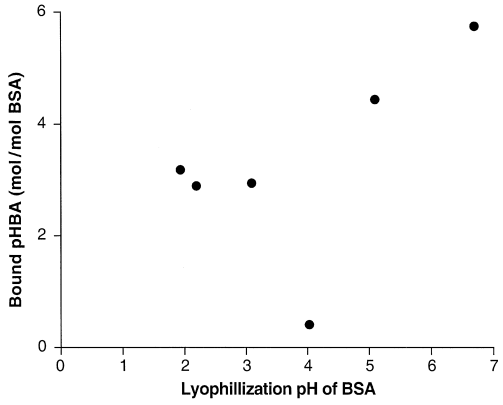

Fig. 2. Dependance of binding of *p*HBA on lyophilization pH of BSA.

in water. A pH of 6.8 was obtained for the imprinted and 3.4 for the non-imprinted protein showing that the HCl was not removed by washing with ethanol. This was the standard extraction procedure for the removal of the template [4]. This result is in agreement with the notion of protein memory [21] that states that a retains the pH of the last solution it was in contact with. Furthermore, when acid adjusted non-imprinted BSA (3.0 mol/mol) was washed with ethanol that contains a small amount of base (triethylamine), to remove the bound acid, it then bound a quantity of ligand (5.5 mol/mol) similar to BSA that had been freeze dried in the absence of any template (5.8 mol/mol). When *p*HBA imprinted BSA was washed with ethanol containing triethylamine, a similar binding result to the above normal ethanol washed BSA was obtained. The *p*HBA binding characteristics of a 1:1 mixture of poly-L-lysine and poly-L-aspartic acid, which approximates BSA in terms of the acid/base ratio, resembled the corresponding imprinted (5.7 mol/mol) or non-imprinted (0.8 mol/mol) BSA preparations. The binding of *p*HBA to either poly-L-lysine (imprinted: 2.0 mol/mol, non-imprinted: 0.13 mol/mol) or poly-L-aspartic acid (imprinted: 0.05 mol/mol, non-imprinted: 0.01 mol/mol) was small.

Scatchard, Langmuir and Freundlich isotherm analysis of the binding data was used to es-

timate *p*HBA binding and kinetics. The two Freundlich constants k and n are related to the capacity of the protein for *p*HBA and to the affinity of the protein for *p*HBA respectively with value of n greater than unity reflecting favourable adsorption conditions [22]. The constant k can have the units of mol/mol when n is unity. As shown in Table 1, it was determined that the Freundlich isotherm offered the best fit to the data on the basis of the derived r^2. This suggests that multiple binding sites with varying affinities are present [23,24].

It is assumed that, if a BSA is specific for a ligand, then the binding of that ligand should be greater than the binding of the same ligand to other BSAs imprinted with similar but different templates. It was observed that irrespective of which template (*o*-, *m*-, *p*-hydroxy, chloro, nitro, etc. benzoic acid) BSA was imprinted with, the quantity of ligand bound was dependent on its pK_a (Fig. 3) rather than structure. This was demonstrated by the observation that binding of a ligand, to its specific BSA is no greater than its average binding to all benzoic acid imprinted BSAs. The implication is that imprinted BSA binds benzoic acids on the basis of their charge rather than on their structure. This finding is in agreement with results obtained from an earlier publication [5].

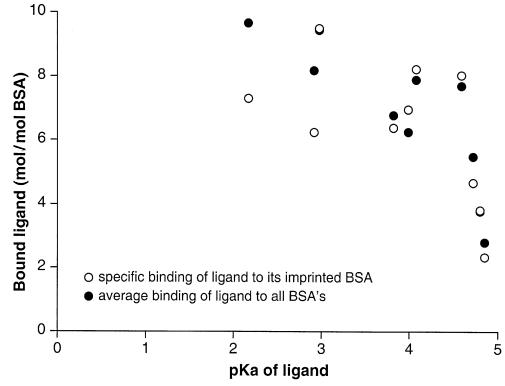

Fig. 3. Binding of benzoic acids to imprinted BSA.

Known active sites in BSA were progressively, irreversibly blocked by incubation in the presence of increasing quantities of FITC. These BSA preparations were then studied for their ability to β-eliminate 4-fluoro-4-(*p*-nitrophenyl)butan-2-one, substrate (**1**), in both aqueous and an organic solvent. The results in the organic solvent were then compared with the ability of other imprinted and non-imprinted BSAs to carry out this reaction.

The initial rate of β-elimination in aqueous solution of (**1**) showed no decrease until after

Table 1
Binding of *p*HBA to imprinted and non-imprinted proteins
na = data not available.
Freundlich isotherm $\ln y = \ln k + 1/n \ln[S]$.
Langmuir–Hinshelwood isotherm $[S]/v = [S]/k + 1/kK$.
Scatchard $y/[L] = n/Kd - y/Kd$.

Template used to imprint BSA	Freundlich		Freundlich	Langmuir/Scatchard
	n	k (mol/mol)	r^2	r^2
*p*HBA	1.1 ± 0.02	6.6 ± 0.07	0.9992	0.8061/0.7087
Jeffamine	1.08 ± 0.04	8.75 ± 0.02	0.9969	0.9746/0.9361
Non-imprinted	1.29 ± 0.02	5.13 ± 0.04	0.9998	0.903/0.8283
HCl pH 5.1	1.4 ± 0.09	3.92 ± 0.18	0.9863	0.9836/0.9387
4.0	0.81 ± 0.30	0.2 ± 0.19	0.7944	na
3.1	0.92 ± 0.09	2.28 ± 0.19	0.9928	na
2.2	1.07 ± 0.05	2.27 ± 0.07	0.9902	0.3994/0.369
1.9	1.11 ± 0.06	2.9 ± 0.1	0.9958	0.7979/0.7033
Imprinted Asp / Lys				
Jeffamine	1.35 ± 0.07	5.66 ± 0.04	0.9831	0.9868/0.9194
Non-imprinted	1.14 ± 0.03	1.22 ± 0.02	0.9242	0.5658/0.0225

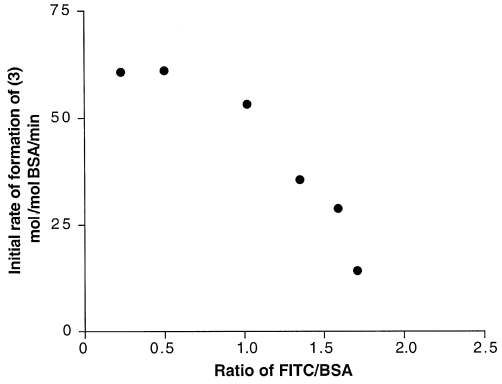

Fig. 4. Dependance of initial rate of β-elimination on FITC/BSA ratio in aqueous solution.

one equivalent of FITC had been bound (Fig. 4). On further FITC binding, the rate tended to zero at a FITC/BSA ratio of 2. When V_{max} and K_m values, derived from Hanes–Woolf graphs, were plotted against FITC/BSA ratios, a similar trend to that shown in Fig. 4 was observed. The use of the polyamino acids, either separately or as a 1:1 mixture in place of BSA, produced low initial rates of reaction and were not investigated further. Also, previous studies with BSA on this reaction have shown that a decrease in the pH of the solution causes a decrease in the reaction rate as would be expected for a base catalysed reaction [13].

The kinetics of this reaction were similarly investigated in anhydrous acetonitrile (Table 2). The BSA preparations freeze dried at different pH showed a decrease in V_{max} as the lyophilization pH was lowered, while BSA preparations blocked with FITC now showed little variation. Similar V_{max} values were obtained with non-imprinted BSA, BSA imprinted with *p*HBA or *N*-isopropyl-4-nitrobenzylamine, (TSA) (**3**), a transition state analogue for the β-elimination reaction that had been used in previous studies [13,18,19]. The BSA imprinted with Jeffamine ED-600 showed a three-fold enhancement in value. Neither poly-L-lysine or poly-L-aspartic acid showed significant catalytic activity but their 1:1 mixture did produce a small increase in initial rate while the imprinted 1:1 mixture produced an initial reaction rate that was similar to imprinted BSA. The K_m values, which were quite large and therefore reflect low affinity, remained fairly constant.

The above results could be concluded as follows. The technique of bio-imprinting appears to alter some of the ionic or salt bridges between surface amine and carboxyl groups so as to increase the number of isolated ionised groups. These bonds have been postulated as stabilising protein structure in organic solvent [25].

This is possibly achieved by the template associating itself with either the charged amine or carboxyl groups thus disrupting the link between them. When the template is removed in the washing process the ionised groups are held apart because of the rigidity of the protein in organic solvents. The primary factor responsible for ligand binding was its pK_a rather than its structure as shown by the results in Fig. 3. The experiments of Dabulis and Klibanov [5] demonstrated a similar effect with carboxylic acids. It is probable that the carboxylate ion, so formed by imprinting, is responsible for the observed binding and catalytic activity. Such a mechanism has been postulated before as carboxylates become more basic in anhydrous organic solvents [26]. This would be in accordance with the concept of using transition state analogues such as *N*-isopropyl-4-nitrobenzylamine (**3**) to generate a catalytic antibody with a carboxylate group positioned adjacent to the acidic proton of 4-fluoro-4-(*p*-nitrophenyl)-butan-2-one (**1**). Further support comes from the recent publication of Menger et al. [27] in which a polymer developed from carboxylic acids and amines, using combinatorial chemistry, was shown to be an effective catalyst for a similar reaction to that shown in Fig. 1.

The addition of water to imprinted BSA, which has been shown to reduce binding [4] and catalytic effect [13] may therefore do so by enabling these ionic bridges to reform, either by increasing the flexibility of the protein or by resolvation of the ions. Likewise, protonation of

Table 2
β-Elimination of (1) by imprinted and non-imprinted proteins
nd = not determined.

Template used to imprint BSA	Initial rate [of (1) at 13 mM] (mol mol^{-1} BSA min^{-1})	K_m (mM)	V_{max} (mol mol^{-1} BSA min^{-1})
*p*HBA	0.228	120.5	2.4
Jeffamine	0.55	113.5	5.75
TSA	0.189	145.4	2.27
Non-imprinted	0.166	145.6	1.91
HC1 pH 5.1	0.156	94.8	1.36
4.0	0.065	106.3	0.67
3.1	0.036	95.5	0.31
2.2	0.012	106.4	0.11
1.9	0.013	39.5	0.05
BSA blocked with FITC			
Ratio 0.2	0.047	35.3	0.2
0.5	0.088	12.2	0.22
1.02	0.069	10.8	0.15
1.35	0.114	23.8	0.38
1.59	0.126	1.7	0.22
1.71	0.137	4.0	0.28
Asp / Lys imprinted with			
Jeffamine	0.325	nd	nd
Non-imprinted	0.061	nd	nd
Lys			
Non-imprinted	0.025	nd	nd
Asp			
Non-imprinted	0.011	nd	nd

the carboxylate ions also causes a decrease in binding and catalytic capacity by converting it into its neutral form. This dependency is shown in Fig. 2 where the quantity of bound *p*HBA decreased as the lyophilization pH decreased. The increase in binding below pH 3.5 was possibly due to the exposure of more carboxyl/amine groups due to the known N–F transformation that BSA undergoes at pH 4.0 [28].

The initial report of imprinting by Braco et al. [4] and Dabulis and Klibanov [5] rely upon the difference in binding of imprinted BSA and an acid adjusted non-imprinted BSA as a control. This control BSA was derived by freeze drying BSA at an acid pH (HCl) similar to the pH of the imprinted protein. As has been shown above, the acid was not removed in the washing process. and the quantity of *p*HBA bound is dependant upon the lyophilization pH. The result was to under estimate the binding capacity of the non-imprinted BSA and hence over estimate the effect of imprinting BSA with a specific template. They do, however, make the point that binding is due to hydrogen bonding which would also be consistent with the present results.

Progressive blocking of two sites in BSA, with FITC, has previously been shown to cause a decrease in the reaction rate, in phosphate buffer, of the base promoted rearrangement of benzisoxazoles to salicylonitriles [20]. This reaction is believed to be catalysed by the lysine groups found in the sites known as Sudlow Site I and Sudlow Site II where the majority of ligand binding, in serum albumins, takes place [29]. The published crystal structure data has confirmed these two sites as cavities [12]. The

present results suggests that the site in BSA most easily accessed by FITC plays little or no part in the β-elimination reaction in aqueous solution. Such an observation is consistent with the accepted view on the nature of these two sites, in that Site I which is an open pocket will bind large, polar molecules while Site II, a tighter pocket binds small non-polar, often aromatic, molecules [29]. In anhydrous acetonitrile, the reaction did not take place in either of the sites referred to above as progressive blocking of them had no effect on the reaction rate. Therefore, it is likely that, as with binding, the observed catalysis with imprinted BSA is derived from charged amino acids found on the surface of the molecule. The Freundlich isotherm data support the above inference that binding was due to isolated charged groups on the protein surface as the affinity value *n* showed a small variation, while the capacity to bind ligand, *k*, changed in a manner that is related to the blocking of these groups.

The observation that only the imprinted 1:1 mixture of poly-L-lysine and poly-L-aspartic acid can act as a catalyst or as an absorbent with similar efficiency to imprinted BSA further strengthens the above assumption. The polyamino acid mixture has no recognised structure but has many of potentially interactive groups. So, if the binding sites on BSA and the polyamino acid mixture are similar there is a possible inference that binding involves mostly basic and acidic groups in combination. The effect of bio-imprinting is therefore to increase the number of unpaired ionic charges on the surface of the protein. This synergistic effect could possibly be utilised by the inclusion of other amino acids, such as serine, into the structure. The techniques of combinatorial chemistry or by the approach of Atassi and Manshouri [30] would allow a number of potentially active sites to be synthesised. The activity of enzymes has been increased by bio-imprinting them with an inhibitor [11]. A similar approach of bio-imprinting the above synthetic active sites, with a suitable template, could then produce an effective catalyst analogous to a proteinase for use in organic solvents.

Acknowledgements

The author is grateful to the Biotechnology and Biological Research Council for the financial support of this work and also to colleagues at IFR for their helpful comments and suggestions, especially Dr. G.W. Niven and Mrs. R. Patel.

References

[1] E.N. Vulfson, in: P. Woolley, S.B. Petersen (Eds.), Lipases: Their Structure, Biochemistry and Application, C.U.P., 1994, pp. 271–288.
[2] A.M. Klibanov, Acc. Chem. Res. 23 (1990) 114.
[3] L. Pauling, Nature 161 (1948) 707.
[4] L. Braco, K. Dabulis, A.M. Klibanov, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 274.
[5] K. Dabulis, A.M. Klibanov, Biotechnol. Bioeng. 39 (1991) 176.
[6] I. Mingarro, C. Abad, L. Braco, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 3308.
[7] A. Johnsson, K. Mosbach, M. Mansson, Eur. J. Biochem. 227 (1995) 551.
[8] J.O. Rich, J.S. Dordick, J. Am. Chem. Soc. 119 (1997) 3245.
[9] M. Stahl, M. Mansson, K. Mosbach, Biotechnol. Lett. 12 (1990) 161.
[10] M. Stahl, U. Jeppsson-Wistrand, M. Mansson, K. Mosbach, J. Am. Chem. Soc. 113 (1991) 9366.
[11] A.J. Russell, A.M. Klibanov, J. Biol. Chem. 263 (1988) 11624.
[12] S. Colonna, A. Manfredi, R. Annunziata, Tetrahedron Lett. 29 (1988) 3347.
[13] C.J. Slade, E.N. Vulfson, Biotechnol. Bioeng. 57 (1998) 211.
[14] D.C. Carter, J.X. Ho, Adv. Protein Chem. 45 (1994) 153.
[15] R.P. Taylor, J. Am. Chem. Soc. 98 (1976) 2684.
[16] K.M. Shokat, C.J. Leumann, R. Sugassawara, P.G. Schultz, Nature 338 (1989) 269.
[17] J.V. Beach, K.J. Shea, J. Am. Chem. Soc. 116 (1994) 379.
[18] R. Muller, L.I. Andersson, K. Mosbach, Makromol. Chem., Rapid Commun. 14 (1993) 637.
[19] Y. Ohya, J. Miyaoka, T. Ouchi, Macromol. Rapid Commun. 17 (1996) 871.
[20] K. Kikuchi, S.N. Thorn, D. Hilvert, J. Am. Chem. Soc. 118 (1996) 8184.
[21] A. Zaks, A.M. Klibanov, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 3192.
[22] X. Zhaoyi, Z. Quanxing, W. Changlong, W. Liansheng, Chemoshere 35 (1997) 2269.
[23] A.J. Ramos, E. Hernandez, Anim. Feed Sci. Technol. 62 (1996) 263.

[24] S. Tunesi, M. Anderson, J. Phys. Chem. 95 (1991) 3399.
[25] D.S. Hartsough, K.M. Merz Jr., J. Am. Chem. Soc. 115 (1993) 6529.
[26] F. Hollfelder, A.J. Kirby, D.S. Tawfik, Nature 383 (1996) 60.
[27] F.M. Menger, J. Ding, V. Barragan, J. Org. Chem. 63 (1998) 7578.
[28] J.F. Foster, in: V.M. Rosenoer, M. Oratz, M.A. Rothschild (Eds.), Albumin Structure, Pergamon, Oxford, 1977, p. 53.
[29] T. Peters, Jr., All about Albumin; Biochemistry, Genetics and Medical Applications, Academic Press, 1996, pp. 76–132.
[30] M.Z. Atassi, T. Manshouri, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 8282.