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MICROENVIRONMENTAL CONTRIBUTIONS TO THE CHROMATOGRAPHIC BEHAVIOR OF SUBTILISIN IN HYDROPHOBIC-INTERACTION AND REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

Genetically engineered variants were used to examine how microenvironmental changes in the S_1 substrate binding subsite of subtilisin contribute to chromatographic behavior of proteins on hydrophobic-interaction chromatography (HIC) and reversed-phase chromatography (RPC) columns. Gradient elution studies over a wide pH range showed that conditions could be found where a HIC support could separate proteins varying by one amino acid. Although all single-site variants could not be separated by HIC, this chromatographic mode was found to be complementary to cation-exchange chromatography for the separation of such variants. RPC was found to be of much less utility in the resolution of variant proteins. Retention and resolution of subtilisin variants was found to vary on RPC with the concentration and type of mobile phase pairing agent.

INTRODUCTION

The physical and chemical properties of amino acids in proteins differ from those found free in solution due to the impact of the surrounding environment. Because of protein folding, side-chains may be surrounded by other amino acids, hydrogen-bonded to solvent molecules, linked to external carbohydrate moieties or combined with any of the above. Specific protein microenvironments are determined by clusters of residues, including those that are consecutively linked and discontinuous neighbors, as well as any surrounding solvent molecules associated with these amino acids. The localized environment strongly influences the pK_a of ionizable side-chains in amino acids. For example, it was shown[1] that the active-site microenvironment in acetoacetate decarboxylase shifted the pK_a of an active-site lysine down to 5.9. This information is especially significant in view of the current advances being made in protein engineering. Studies investigating single amino acid contributions to

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protein thermostability[2], folding[3], substrate affinity[4], catalysis[5] and antibody epitope recognition[6] using site-directed mutagenesis have already been reported. The role of amino acid contributions to protein chromatographic behavior is examined here using genetically engineered proteins.

The protein, subtilisin, is a well characterized, single-chain serine protease of 27 500 daltons from *Bacillus amyloliquefaciens*. It is an attractive model protein because the gene has been cloned[7] and there is a large amount of data available on structural variants of the enzyme[8]. An X-ray crystallographic analysis at 1.8 Å resolution provided strong evidence that the three-dimensional structure of the wild-type enzyme is conserved in the engineered variants[9]. A recent paper describing subtilisin retention using immobilized-metal affinity chromatography reported that substituted amino acid residues, as far away as 14 Å, affected the interaction between the active site histidine and the sorbent enough to alter chromatographic behavior[10]. In another study, it was reported that two variants differing by a single methylene group were separated using cation-exchange chromatography (CEC)[11]. Although many variants were resolved using these two chromatographic modes, not all subtilisin variants could be successfully separated.

Protein separations based on surface hydrophobicity are accomplished on either hydrophobic-interaction or reversed-phase supports, depending on the method of elution. To further explore the influence of substituted residues and their proposed effect on highly structured water, an investigation probing hydrophobic interactions was initiated. This study examines the role of amino acid contribution to subtilisin retention in the S_1 subsite microenvironment by both hydrophobic-interaction chromatography (HIC) and reversed-phase chromatography (RPC).

HIC was originally introduced (in the early 1970s[12-14]) for protein separations using low ligand density alkyl- or arylagarose sorbents and a descending salt gradient. Adsorption is achieved at high concentrations of salt, with elution generally obtained using a descending ionic strength gradient ranging from several molar salt to dilute buffer. The possibility of denaturing a protein during this process is not much greater than in an ammonium sulfate fractionation. As a result, HIC has been shown to be a non-denaturing chromatographic method[15]. Retention studies have shown selectivity and efficiency for protein separations in HIC to be most favorable with an ammonium sulfate mobile phase[16-18].

Using natural occurring lysozyme variants, Fausnaugh and Regnier[19] reported the existence of a chromatographic contact surface area between the proteins and the HIC sorbent. The substitution of a hydrophilic amino acid within this contact region was observed to decrease protein retention. Recent studies have reported resolution of polypeptides with tertiary structure using gradient elution on HIC and RPC supports[20,21]. These polypeptides were designed and synthesized to differ from each other by single-residue replacement or deletion. Both studies concluded that tertiary structure plays a prominent role in the HIC retention mechanism and that differences in solute selectivity, between the two modes, were attributed to alterations in the three-dimensional structure of the polypeptides resulting from the standard mobile phase conditions used in RPC. A similar conclusion was discussed in a comparison of protein separations using HIC and RPC[22]. To further probe the selectivity of HIC versus RPC, the subtilisin variants were examined for their chromatographic behavior on a RPC column.

The separation of protein and peptide mixtures on reversed-phase supports is widely practiced[23,24]. Because the mobile phase composition varies enormously, the elution process in RPC is completely different from that in HIC, even though both chromatographic modes utilize hydrophobic interactions to retain proteins. In RPC, the weak mobile phase usually consists of an aqueous acidic solution [0.1% trifluoroacetic acid (TFA)], while the eluting mobile phase is an organic solvent (methanol, isopropanol or acetonitrile). Because the number of solvent molecules (concentration of organic solvent) needed to elute the protein may be accurately measured, the stoichiometric displacement model can be applied to quantitate the interactions between protein and sorbent stationary phase[25,26]. Kunitani et al.[27] have taken this approach to help explain the differences in retention for thirty variants of interleukin-2 using gradient elution on a reversed-phase support. It was concluded that stoichiometry factors determined from RPC retention data (1) corresponded to changes in the relative contact area between solute and stationary phase for the different variants and (2) detected conformational changes, in the less stable variants, resulting from a higher degree of unfolding due to the elution conditions. These studies suggest that a RPC probe should provide quantitative data, as did CEC, regarding any changes in the S_1 subsite affecting subtilisin chromatographic behavior. For this reason, a study was conducted to determine if any amino acid substitutions affected the chromatographic behavior of subtilisin in RPC and the results were compared with those obtained using HIC.

EXPERIMENTAL

Proteins and reagents

Bacillus amyloliquefaciens subtilisin wild-type and site-directed variants were a generous gift of Genencor (South San Francisco, CA, U.S.A.). These proteins were used without further purification. All reagents were of AR grade or comparable quality.

To prevent autolysis, proteins were stored in 50% aqueous propylene glycol–5mM calcium chloride–10mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (pH 7.6) at $-12°C$. Protein concentrations were approximately 10 mg/ml. Enzymatic activity of collected fractions was determined from a modified progress-curve analysis[28].

Equipment

Protein retention measurements were made with a Varian 5000 chromatographic system, equipped with a UV-100 detector (Walnut Creek, CA, U.S.A.) and a Valco Model C6U injector with a 15-μl sample loop (Houston, TX, U.S.A.). Protein absorbance was monitored at 280 nm and eluent conductance was monitored with an Anspec AN400 ion chromatograph (Ann Arbor, MI, U.S.A.). Data were collected on a Kipp & Zonen BD41 dual-channel chart recorder (Delft, The Netherlands).

Mobile phase

Mobile phase buffers used in HIC were chosen so that the buffer pK_a was within 1 pH unit of the desired eluent pH: sodium acetate (Mallinckrodt, Paris, KY, U.S.A.) (pH 5.0); 2-(N-morpholino)ethanesulfonic acid (MES) (Calbiochem-Behring, LaJol-

la, CA, U.S.A.) (pH 6.0); TES (Sigma, St. Louis, MO, U.S.A.) (pH 7.0 and 8.0); sodium borate (Mallinckrodt) (pH 9.0). Buffer A contained a final concentration equivalent to 0.01 M buffer B and 1.3 M ammonium sulfate (Grade III, Sigma). Buffer B was a 0.01 M aqueous solution comprised of the appropriate salt for the pH range studied. All buffers were adjusted to the desired pH at room temperature with either hydrochloric acid or sodium hydroxide.

Solutions used for RPC were prepared without adjustment in pH. Two acids were employed in the mobile phase eluents. In the first, buffer A was 0.1% TFA (99% purity, Pierce, Rockford, IL, U.S.A.) in water. Buffer B was 0.1% TFA in acetonitrile (HPLC grade, American Burdick & Jackson, Muskegon, MI, U.S.A.). The second set of conditions simply substituted 20% formic acid (96% purity, Aldrich, Milwaukee, WI, U.S.A.) for the 0.1% TFA in both buffer A and buffer B.

All aqueous solutions were prepared using deionized water. All solutions were prepared fresh daily, filtered through a Raining Nylon-66 (0.45-μm) filter (Woburn, MA, U.S.A.) and deaerated prior to use.

Chromatography

HIC was performed on a hydrophilic polymer-based (1000 Å pore size, 10 μm particle size) TSKgel Phenyl-5PW column (Toyo Soda, Yamaguchi, Japan), with dimensions of 7.5 cm × 0.75 cm I.D. and a 0.1 mmol/ml ligand density. Samples were examined by gradient elution with a 20-min linear descending salt gradient at a flow-rate of 1 ml/min. Retention maps, for the pH range 5.0–9.0, were assembled from data collected in this manner. Gradient times were later modified to optimize variant separations. The solvent perturbation peak observed by both the UV and conductance detectors was taken as the retention time for unretained protein (t_0).

RPC was performed on a poly(styrene–divinylbenzene) support (1000 Å pore diameter, 8 μm particle size) PLRP-S column (Polymer Labs., Church Stretton, U.K.) with dimensions of 5.0 cm × 0.41 cm I.D. All retention measurements were made at a flow-rate of 1 ml/min using a non-linear gradient protocol: 0–5 min, 0–20% buffer B; 5–30 min, 20–75% buffer B.

All samples in both modes were chromatographed in duplicate with injection volumes varying between 5 and 70 μl (volume was constant for any one analysis) depending on the variant.

RESULTS AND DISCUSSION

Hydrophobic-interaction chromatography

Many proteins interact with both ion-exchange and hydrophobic-interaction supports, due to heterogenous surfaces comprised of solvent accessible charged and non-polar amino acids. The chromatographic behavior of a protein is determined by the number of interactions and the corresponding affinity between surface residues and the sorbent stationary phase. Even though there are no obvious hydrophobic clusters, many residues considered to be hydrophobic do reside on the surface of subtilisin: all three tryptophanes, all three phenylalanines, all ten tyrosines, one out of five methionines, three out of eleven isoleucines, five out of ten leucines, six out of twenty-four valines, nine out of twenty-eight alanines and nine out of fourteen pro-lines[29]. From these structural data, it was expected that subtilisin would be retained

on HIC sorbents. In addition, the reported low-ionic-strength mobile-phase (less than 150 mM), needed to displace subtilisin variants from a cation-exchange sorbent[11], also suggested effective retention using HIC.

Initially, three columns were chosen to determine which stationary phase ligand best resolved the subtilisin variants. The supports were of similar physical dimensions, but differed in type of hydrocarbon ligand (alkyl chain versus aryl ring). Peak shape and column efficiency were found to be superior on the TSKgel Phenyl-5PW column compared to either the Synchropak-Propyl or Synchropak-Pentyl supports.

Retention maps. The subtilisin variants used throughout this study are listed in Table I. To select the optimum conditions for variant resolution, a retention map charting retention time *versus* mobile phase pH was assembled. Recently it was re-

TABLE I

SUBTILISIN RESIDUE SUBSTITUTIONS

Variant	Amino acid/position in wild type	Substitution	Variant	Amino acid/position in wild type	Substitution
Multiple substitutions			Single substitutions		
FQK	Met-50	Phe	D166	Gly-166	Asp
	Glu-156	Gln	E166	Gly-166	Glu
	Gly-166	Lys	S166	Gly-166	Ser
FQK R275	Met-50	Phe	N166	Gly-166	Asn
	Glu-156	Gln	C166	Gly-166	Cys
	Gly-166	Lys	M166	Gly-166	Met
	Gln-275	Arg	H166	Gly-166	His
FQK R170	Met-50	Phe	K166	Gly-166	Lys
	Glu-156	Gln	R166	Gly-166	Arg
	Gly-166	Lys	P166	Gly-166	Pro
	Lys-170	Arg	V166	Gly-166	Val
FQK R43	Lys-43	Arg	Y166	Gly-166	Tyr
	Met-50	Phe			
	Glu-156	Gln			
	Gly-166	Lys			
FQK R27-R43	Lys-27	Arg			
	Lys-43	Arg			
	Met-50	Phe			
	Glu-156	Gln			
	Gly-166	Lys			
FQK Multi	Tyr-21	Phe			
	Lys-27	Arg			
	Lys-43	Arg			
	Met-50	Phe			
	Glu-156	Gln			
	Gly-166	Lys			
	Lys-170	Arg			
	Gln-275	Arg			
QK	Glu-156	Gln			
	Gly-166	Lys			
QD	Glu-156	Gln			
	Gly-166	Asp			
SD	Glu-156	Ser			
	Gly-166	Asp			

ported that according to the mode of separation, different regions of a protein surface determine chromatographic behavior[30]. Therefore, the initial retention maps were generated with the multiple substitution variants to determine whether position 166 was included in the subtilisin HIC "footprint" region. Results of the FQK Series retention map are found in Fig. 1. The FQK variants preserve a similar curve shape distinct from that found for wild-type subtilisin and elute before the wild type at all pH values (except FQK R27-R43 at pH 5.0). Protein retention decreased, between pH 9.0 and 6.0, then suddenly increased at pH 6.0 for all variants including the wild type. Mobile phase pH influences retention in HIC by altering the solute surface charge. By probing protein hydration, known to be affected by charged groups, it was shown that water molecules were selectively arrayed in the vicinity of ionic regions and that the strength of water interactions were in the order water–anion > water–cation > water–dipole[31]. Therefore, as His 64, the active-site histidine, becomes ionized with the decrease in pH, water is attracted to the active site and subtilisin affinity towards the HIC support diminishes. This clearly explains the retention trend between pH 6.0 and 9.0.

The elution order for the FQK series variants compared to the wild type can be explained using a similar argument. The substitutions at positions 156 and 166 (found in all the variants shown in Fig. 1) increase the net surface charge by +2. The additional subsitution at position 275 further increases the net charge to +3, compared to the wild-type enzyme. Therefore, these variants, having a greater surface charge than the wild type, are less hydrophobic and elute from the HIC column earlier in the gradient protocol. To better identify the role of position 166 in HIC, the 156/166 double substitution variants were next investigated (Fig. 2).

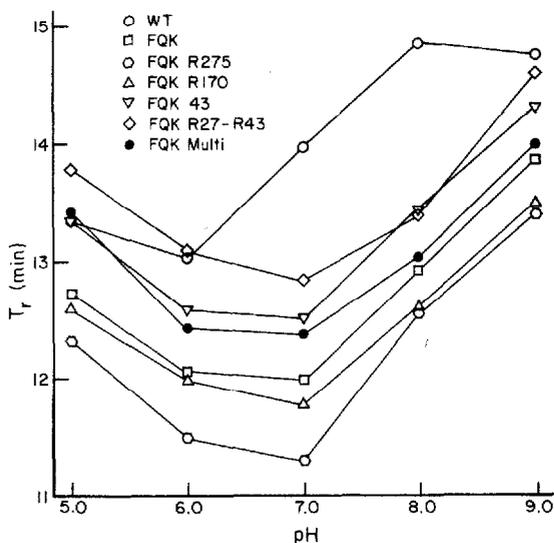The curve shape for the double-mutation variants was similar to that observed

Fig. 1. HIC retention map of wild-type subtilisin and FQK series variants. The retention time (T_r) is plotted against mobile phase pH using a 20-min linear gradient from 1.3 to 0 M ammonium sulfate at a flow-rate of 1 ml/min on a TSKgel Phenyl-5PW column.
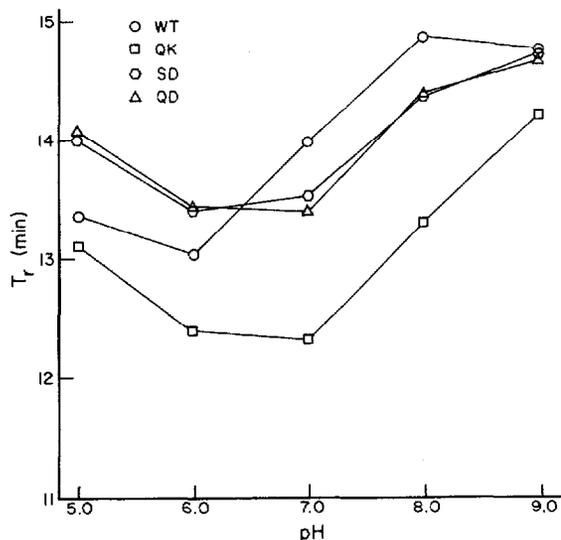
Fig. 2. HIC retention map of wild-type subtilisin and double-mutation variants. The retention time is plotted against mobile phase pH using a 20-min linear gradient from 1.3 to 0 M ammonium sulfate at a flow-rate of 1 ml/min on a TSKgel Phenyl-5PW column.

for the FQK series; however, the selectivity differed, with both SD and QD being retained longer than the wild type below pH 7.0. This, combined with the QK variant eluting prior to wild-type subtilisin at every pH, confirms that substitution at position 166 affects the chromatographic contact area microenvironment. Further, comparison of the SD and QD variant retention data revealed that position 156 had a minimal effect on HIC retention, unlike the analysis reported earlier[11], where a significant difference in cation-exchange retention was attributed to position 156 at pH 5.0. These findings support the earlier claim that surface region recognition is dependent on the chosen chromatographic mode. An interesting aside showed the QK variant consistently eluted 0.35 min later than the FQK variant at all mobile phase pH values, suggesting the Met-to-Phe substitution at position 50 slightly affects the chromatographic behavior of subtilisin in HIC.

Enzymatic recovery of over 90% was recorded for collected fractions from every pH. However, the activity assay for variants eluted at pH 5.0 was anomalous. Although over 90% recovery was observed, it took far longer for the enzymatic reaction to come to completion compared to samples taken at other pH values. At first, this was believed to be a result of the near-total enzymatic inhibition that takes place at pH 5.0 (see ref. 11). Attempts made to compensate in the assay for the acidic mobile phase pH had no effect. A plausible explanation comes from a review of the retention maps. Here it was noticed that retention for all subtilisin variants also increased at pH 5.0. It is known that changes in protein secondary or tertiary structure can have a major impact on chromatographic retention in HIC[32]. From the cation-exchange experiments, protein unfolding and eventual denaturation was found to occur below pH 5.0. A recent study revealed that conformational changes, as well as aggregate formation, appear to be functions more of the mobile phases than of the HIC stationary phase[33]. Therefore, the combined mobile phase conditions of

acidic pH and high salt concentration possibly led to partial unfolding or a conforma-
tional change in subtilisin. A greater protein contact surface area, associated with the
conformational change, would have been exposed to the HIC sorbent, explaining the
longer retention time observed at pH 5.0. Since activity was eventually restored for
fractions collected at this pH, the partial unfolding was not severe enough to denature
subtilisin. Consequently, the altered enzymatic activity profile appears to have result-
ed from the slow kinetic process associated with protein refolding, because full en-
zymatic recovery was obtained from these fractions after sufficient equilibration at
pH 8.0.

Position 166 variants. From Fig. 2, a difference in selectivity was observed
between an aspartate and lysine when inserted at position 166. Neither charged resi-
due should directly interact with the hydrophobic stationary phase, yet their presence
altered the microenvironment enough to effect retention. The position 166 variants
were separated into two groups: the first contained the charged amino acid sub-
stitution variants and the second was composed of the remaining neutral residue
replacements. Retention maps were derived to reveal the most favorable mobile phase
pH for variant separation (Figs. 3 and 4). With the exception of D166 and E166, all
the position 166 variants conserved the retention curve established by the wild type
and maintained elution order throughout the pH spectrum. Because no current meth-
od is available to quantitate the interactions between solute and sorbent for HIC, it is
difficult to ascertain the circumstances behind the retention irregularity found with
the negatively charged substitutions. Fig. 3 shows similar curve shape for both var-
iants. In an investigation on the role of hydrogen bonding and biological specificity,
Fersht *et al.*[34] revealed the following representative values of hydrogen bonding ener-
getics for certain donor/acceptor pairs: water/water, -6.4 kcal mol^{-1}; water/meth-
anethiol, -3.1 kcal mol^{-1}; imidazolium/water, -14 kcal mol^{-1}; acetate/water,
-19.8 kcal mol^{-1}. This trend supports the earlier reported study[31] regarding associ-
ation of water with ionic species and also supports the elution order presented in the
retention maps. Perhaps the substitution of a negative charge at position 166 influen-
ces the water affinity within the microenvironment, and rather than allowing the
entropic displacement of water with an increase in salt concentration, the energetical-
ly favorable water/anionic pair remains intact and thus the D166 and E166 variants
become inherently less hydrophobic.

Separations were evaluated according to the detected maximum differences in
variant retention at pH 8.0 (Figs. 3 and 4). Optimization of gradient elution condi-
tions disclosed that better resolution was attained when protein samples were applied
to the HIC support using an ammonium sulfate concentration of 2.0 M in 0.01 M
buffer and using a linear gradient of 60 or 90 min. The first separation attempt
focused on discriminating between three types of contrasting substitutions: a charged
residue, a polar neutral residue and a non-polar neutral residue (Fig. 5). As was the
case in CEC, proteins incorporating all three types of amino acid substitutions were
resolved, verifying the importance of microenvironment perturbations that indirectly
contribute to protein retention (the glutamate side-chain should not interact directly
with the phenyl-linked stationary phase). The sensitivity of HIC to minor changes in
protein structure was further demonstrated by the ability to detect a change in hydro-
phobicity between an asparagine and methionine (Fig. 6). The strength of water
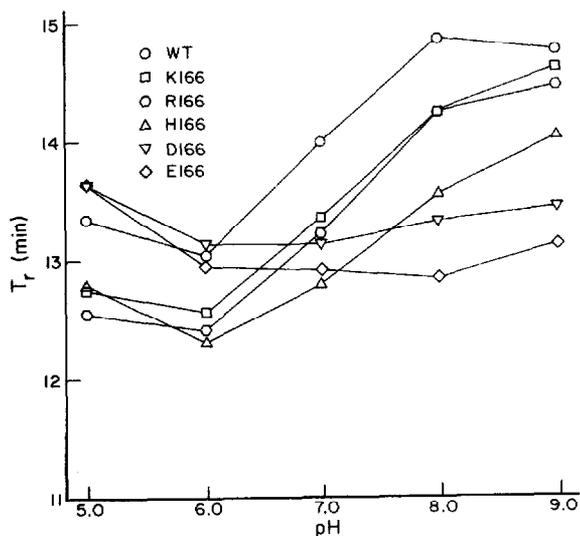hydration apparently differs for these two residues at this location. Fig. 7 reveals the

Fig. 3. HIC retention map of wild-type subtilisin and position 166 charge substitution variants. The retention time is plotted against mobile phase pH using a 20-min linear gradient from 1.3 to 0 M ammonium sulfate at a flow-rate of 1 ml/min on a TSKgel Phenyl-5PW column.
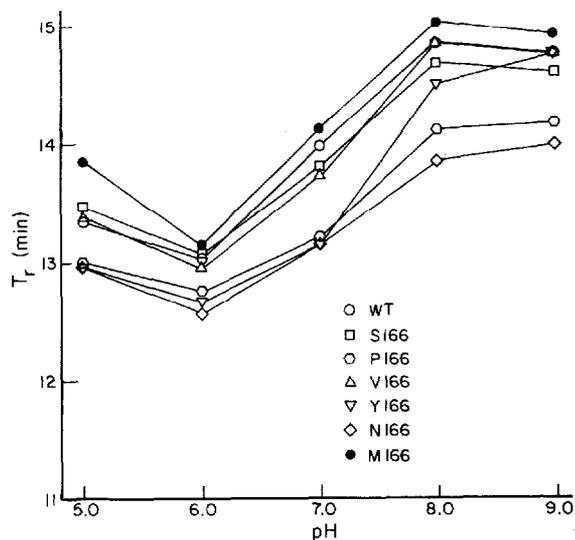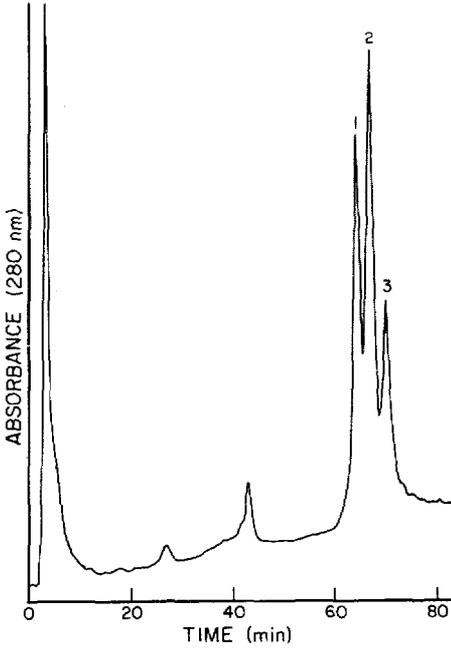

Fig. 4. HIC retention map of wild-type subtilisin and position 166 neutral substitution variants. The retention time is plotted against mobile phase pH using a 20-min linear gradient from 1.3 to 0 M ammonium sulfate at a flow-rate of 1 ml/min on a TSKgel Phenyl-5PW column.

Fig. 5. HIC separation of a mixture of negatively charged, polar neutral and non-polar neutral position 166 substitution variants. Column: TSKgel Phenyl-5PW. Mobile phase: 2.0 M ammonium sulfate in 0.01 M TES (pH 8.0); 90-min linear gradient; flow-rate 1.0 ml/min; Peaks: 1 = E166; 2 = N166; 3 = M166.
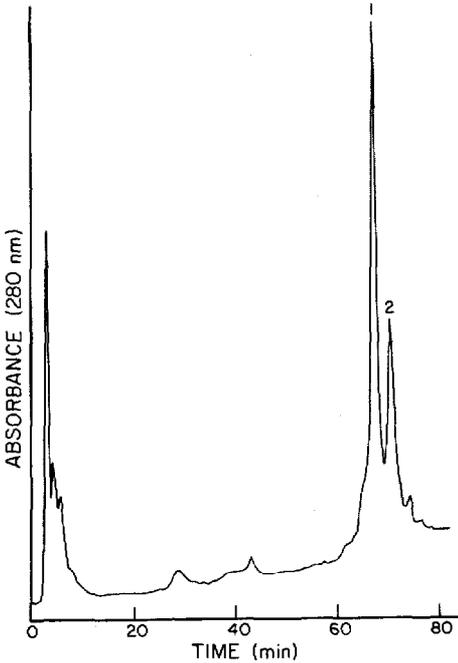

Fig. 6. HIC separation of two neutral substitution position 166 subtilisin variants. Column: TSKgel Phenyl-5PW. Mobile phase: 2.0 M ammonium sulfate in 0.01 M TES (pH 8.0); 90-min linear gradient; flow-rate 1.0 ml/min. Peaks: 1 = N166; 2 = M166.
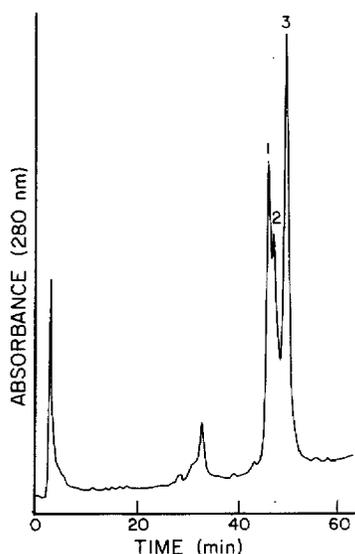
Fig. 7. HIC separation of negatively and positively charged position 166 substitution variants from wild type. Column: TSKgel Phenyl-5PW. Mobile phase: 2.0 M ammonium sulfate in 0.01 M TES (pH 8.0); 60-min linear gradient; flow-rate 1.0 ml/min. Peaks: 1 = E166; 2 = H166; 3 = WT.

chromatographic separation of E166, H166 and the wild type. Good resolution between H166 and wild-type subtilisin exists, with the two charged amino acid substitution variants only slightly separatd from each other. The elution pattern agrees with the predicted order based on the strength of hydration trend.

A comparison of HIC and CEC[11] finds that H166 and wild type have reversed their elution order. Separation of P166 from wild type (Fig. 8) also demonstrates a change in selectivity between the two chromatographic modes (P166 eluted after wild-type subtilisin in CEC). To establish the complementary selectivity for these two chromatographic modes, an attempt to separate variants which coeluted in CEC was made. Fig. 9 shows the separation of N166 from S166 and P166 from V166, completing the HIC resolution of all neutral variants which coeluted in CEC. The elution order established in the retention maps and subsequent separations of position 166 variants corresponds well with the proposed amino acid hydration found for the substituted residues (Table II). Thus, this chromatographic method is able to detect minute changes in the surface hydrophobicity of the S_1 subsite microenvironment due to the substitution of single amino acids at positon 166. Combination of the gradient elution results from the cation-exchange investigation with those presented here reveals the discriminatory power of the two chromatographic techniques, which resolve eleven of the twelve position 166 single-mutation variants.

Reversed-phase chromatography

The next mode used to probe the effects of site-directed mutagenesis on subtilisin was RPC. Along with the established application of the stoichiometric displacement model to protein retention in RPC, this procedure allows the examination of differences in selectivity between the two hydrophobic interaction-driven modes.
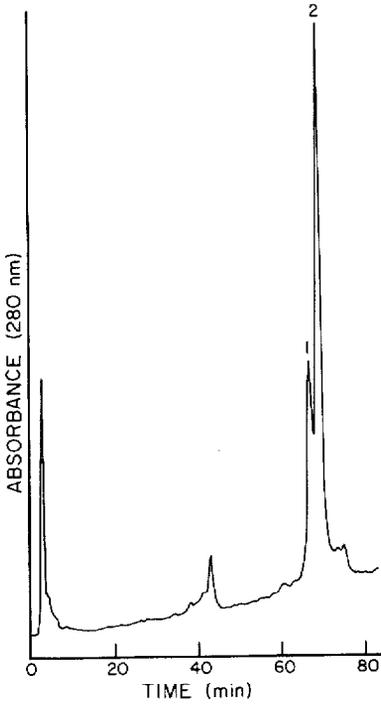
Fig. 8. HIC separation of the P166 variant from wild-type subtilisin. Column: TSKgel Phenyl-5PW. Mobile phase: 2.0 M ammonium sulfate in 0.01 M TES (pH 8.0); 90-min linear gradient; flow-rate 1.0 ml/min. Peaks: 1 = P166; 2 = WT.
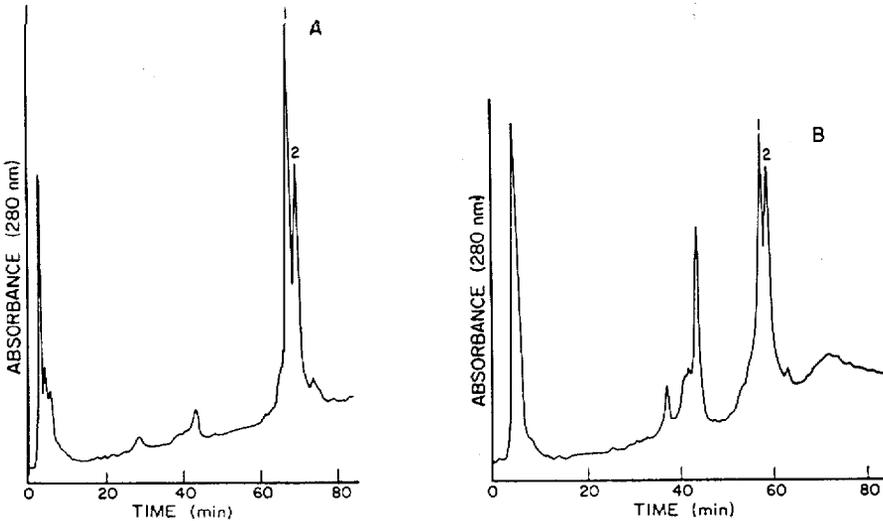

Fig. 9. HIC separation of (A) polar neutral position 166 substitution variants and (B) non-polar neutral position 166 substitution variants. (A) Column: TSKgel Phenyl-5PW. Mobile phase: 2.0 M ammonium sulfate in 0.01 M TES (pH 8.0); 90-min linear gradient; flow-rate 1.0 ml/min. Peaks: 1 = N166; 2 = S166 (B) Column: TSKgel Phenyl-5PW. Mobile phase: 2.0 M ammonium sulfate in 0.01 M TES (pH 8.0); 60-min linear gradient; flow-rate 0.5 ml/min. Peaks: 1 = P166; 2 = V166

TABLE II

PROPOSED AMINO ACID HYDRATION BASED ON NUCLEAR MAGNETIC RESONANCE STUDIES OF POLYPEPTIDES[35]

Amino acid residue	Bound water (mol water per mol amino acid)
Glutamate	7
Aspartate	6
Histidine	4
Lysine	4
Arginine	4
Proline	3
Tyrosine	3
Serine	2
Valine	1
Glycine	1
Methionine	1

The unfavorable facet of this method, however, was the probable loss of enzymatic activity associated with using RPC. The strategy prior to this inquiry was to use chromatographic probes to detect protein surface modifications while maintaining biological activity. Nevertheless, RPC was examined as an analytical method for the investigation of amino acid contributions to the subtilisin S_1 microenvironment.

The use of 0.1% TFA as a pairing agent precluded the necessity for the construction of a pH retention map, because the mobile phase would be held constant at an acidic pH. Although the function of this mobile phase additive is to solubilize proteins, the imposed acidic pH virtually insured denaturation of the acid-labile subtilisin. Before initiation of the isocratic analyses, gradient elution experiments were performed to survey any differences in variant retention. The observed peak shape was sharper than that found in HIC (Fig. 10). Unfortunately, the selectivity was poor. Table III shows the RPC retention results for all the subtilisin variants. No differ-
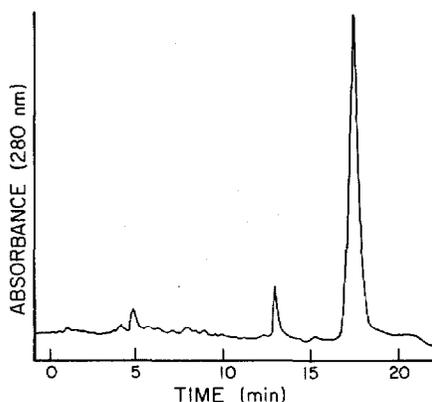

Fig. 10. RPC gradient elution profile for wild-type subtilisin (WT). Column: PLRP-S 1000. Mobile phase: 0.1% TFA in water or acetonitrile; flow-rate 1 ml/min.

TABLE III

REVERSED-PHASE CHROMATOGRAPHY VARIANT RETENTION TIMES GRADIENT ELU-
TION ANALYSIS

0.1% TFA pairing agent.

Variant	Retention time (min)
Wild type	14.54
D166	14.55
E166	14.50
N166	14.48
S166	14.58
M166	14.63
H166	14.60
R166	14.50
K166	14.55
P166	14.70
V166	14.78
Y166	14.70
SD	14.50
QD	14.40
QK	14.30
FQK	14.35
FQK R275	14.30
FQK R170	14.40
FQK R43	14.40
FQK R27-R43	14.45
FQK Multi	14.43

ences were observed. An attempt to separate the two variants with the largest appar-
ent difference, QK and V166, proved unsuccessful. No enzymatic activity for collect-
ed fractions was found, most likely due to irreversible denaturation caused by the
chromatographic condition utilized in RPC. Thus, subtilisin three-dimensional struc-
tural integrity was lost and with it the ability for single residue changes to affect
retention.

A new paring agent was used in an effort to further explore possible variant
selectivity on reversed-phase supports. Formic acid has recently been shown to affect
protein retention in RPC[36]. Depending on the mobile phase acid concentration,
unique structural changes emerge. While no changes in retention for any variants
were recognized, a curious chromatographic anomaly was identified. A comparison
of gradient-eluted FQK R43 using 0.1% TFA and 20% formic acid revealed better
efficiency for the TFA (as judged by narrower peak shape), but improved selectivity
with the formic acid elution as witnessed by the resolution of an autolysis product
(Fig. 11). No activity was recorded for eluted protein.

Although RPC seemed to be a good chromatographic technique for the exam-
ination of amino acid contribution to subtilisin retention, the caveat of this approach
is that protein denaturation, associated with sample interaction with the reversed-
phase mobile phase, may change the three-dimensional structure and concomitantly
alter the chromatographic behavior. It has been observed in several RPC studies that
the combination of a strongly hydrophobic packing material with mobile phases rich
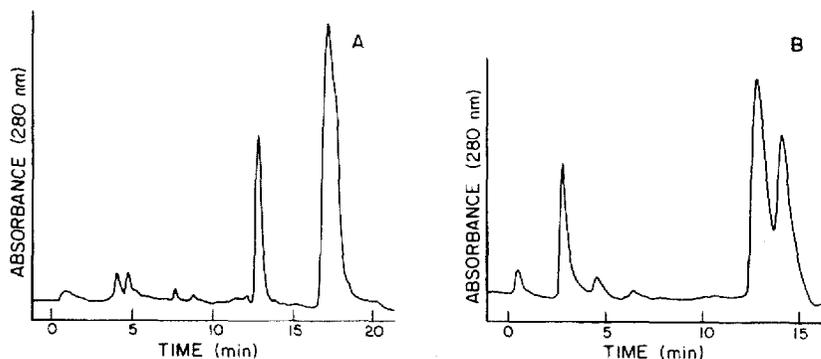
Fig. 11. RPC gradient elution profiles for FQK R43 using (A) 0.1% TFA and (B) 20% formic acid as the aqueous acid ion-pairing agent. Column: PLRP-S 1000. Mobile phases: 0.1% TFA in water or acetonitrile (A) and 20% formic acid in water or acetonitrile (B); flow-rate 1 ml/min.

in organic solvent leads to the denaturation of protein solutes[37-39]. Such disruption in protein tertiary structure seeds conformational changes which alter the chromatographic contact region of the protein. Depending on the severity of unfolding, a surface modification in the native protein may no longer be in an accessible region of a sample applied to a reversed-phase sorbent. In the case of subtilisin, the entire surface domain containing the S_1 microenvironment was probably structurally altered and hence no longer chromatographically accessible. This then negated any effect of the single substitutions at position 166. Even if the area around position 166 was left intact, the disruption of tertiary structure in other parts of the molecule made discrimination based on single-residue substitutions impossible.

CONCLUSION

Resolution of subtilisin site-specific variants was accomplished on the HIC support. From the constructed retention map, pH 8.0 was chosen as the ideal mobile phase pH for the separation study. The absolute differences in retention time did not approach those found earlier in CEC[11]. However, HIC complemented CEC by resolving the neutral substitution variants that coeluted in that chromatographic mode. Analysis of amino acid contribution to hydrophobic interaction was not performed due to the unavailability of a reliable quantitative procedure for HIC.

In contrast to the impressive results in the HIC retention study, the RPC data were disappointing. No separations were realized, even with the multiple-substitution variants. One explanation includes the possibility that none of the mutation sites were located within the RPC contact region. The most likely explanation, however, is that irreversible denaturation occurred upon sample injection, leading to the formation of a totally different chromatographic contact area featuring the normally internalized hydrophobic residues. This could sterically remove position 166, along with a majority of the surface residues associated with the native structure, from possible interaction with the stationary phase ligands.

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