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ANALYSIS OF THE CATALYTIC MECHANISM OF JUVENILE HORMONE ESTERASE BY SITE-DIRECTED MUTAGENESIS

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Abstract—1. Juvenile hormone esterase (JHE) is a serine hydrolase selective for hydrolysis of the conjugated methyl esters of insect juvenile hormones.

2. We have investigated the mechanism of catalytic action of this enzyme by site-directed mutagenesis of the cloned enzyme and expression of the mutants in a baculovirus system.

3. A series of individual mutations of JHE were made to residues possibly involved in catalysis of juvenile hormones, and which are highly conserved in both esterases and lipases.

4. Mutation of the serine residue at position 201 to glycine (S201G), or aspartate 173 to asparagine (D173N), or histidine 446 to lysine (H446K), removed all detectable activity and these mutagenized enzymes were determined to be at least 10^6-fold less active than wild type JHE.

5. Mutation of arginine 47 to histidine (R47H) decreased but did not abolish activity, with K_m essentially unchanged at 66 nM for R47H compared to 34 nM for wild type JHE.

6. The k_{cat} for R47H was decreased from 103 min^{-1} for wild type JHE to 1.9 min^{-1}.

7. In addition, glutamate residue 332 was altered to glutamine (E332Q) and expressed in an *Escherichia coli* system.

8. This mutation was also found to remove all detectable activity.

9. From the results presented in this study and by comparison of JHE to other serine esterases and lipases, we predict that JHE possesses a Ser_{201}–His_{446}–Glu_{332} catalytic triad.

10. In addition, aspartate 173 and arginine 47 are essential for the efficient functioning of JHE.

INTRODUCTION

Numerous enzymes capable of hydrolyzing organic esters have been identified, but an unequivocal physiological role for only two of these enzymes has been established, juvenile hormone esterase and acetylcholinesterase. Juvenile hormone esterase hydrolyzes the highly stable conjugated methyl esters of the insect juvenile hormones and is essential for successful lepidopteran metamorphosis (Hammock *et al.*, 1987; Sparks and Hammock, 1980; Jones *et al.*, 1981). Since this enzyme scavenges very low concentrations of substrates, it is characterized by a moderate k_{cat} and a very high affinity for juvenile hormone. These properties make it suitable for removing trace quantities of juvenile hormone prior to pupation.

For many years it has been assumed that serine esterases hydrolyze substrates by a mechanism analagous to the thoroughly studied serine proteases using a catalytic triad originally proposed for chymotrypsin. However Hanzlik *et al.* (1989) noted that the sequences of three cDNA's coding for JHE, and the messages of other esterases, failed to have conserved amino acids corresponding to the Ser–His–Asp triad of chymotrypsin.

Analysis of JHE by alignment to other esterase protein sequences indicated that in addition to a serine at position 201, which lies in a conserved motif of G × S × G, there is a single conserved His residue at position 446 and Gibney *et al.* (1990) have indicated that His_{440} is probably a catalytic amino acid in the acetylcholinesterase from *Torpedo californica*. A strongly conserved Asp residue is present at position 173 of JHE and Cooke and Oakeshott (1989) have predicted Asp_{160} as a catalytic residue in esterase-6 of *Drosophila melanogaster*. These data suggested the possibility that a Ser_{201}–His_{446}–Asp_{173} catalytic triad may occur in JHE. Hanzlik *et al.* (1989) also predicted a role for Arg_{47} of JHE as an alternative to His in the catalytic mechanism of esterases.

To test these hypotheses, we have carried out site-directed mutagenesis of residues Arg_{47}, Asp_{173}, Ser_{201} and His_{446} of JHE. In addition, lysine residues 29 and 522 were changed to arginine as part of another study and used as controls for analysis of mutagenized JHE. The mutant enzymes were expressed in a baculovirus system and analyzed for kinetic activity to determine if a Ser–His–Asp cata-

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lytic triad may occur in JHE. The JHE sequence was also compared to the recently published structures for the lipase of *Geotrichum candidum* (Schrag *et al.*, 1991) and the acetylcholinesterase of *T. californica* (Sussman *et al.*, 1991) to determine if the motifs examined above could be associated with related structural information. This comparison implicated Glu_{332} as important for the catalytic activity of JHE and based on this observation $glutamate_{332}$ was altered to glutamine (E332Q) and the mutant enzyme compared to wild type JHE when expressed in an *E. coli* system.

MATERIALS AND METHODS

Identification of conserved residues

Using the alignment of esterases published by Hanzlik *et al.* (1989) as a base, additional esterases and lipases were aligned by eye at the regions implicated as being essential for JHE function (Table 1). Based on the high level of conservation in these regions, targetted amino acid residues were chosen as likely candidates for analysis by site-directed mutagenesis.

Site-directed mutagenesis of JHE

The JHE clone used in this study was clone 3hv16B described by Hammock *et al.* (1990), which contains the 1734 bp coding region but not the 3′ untranslated region of JHE cDNA. This clone was in the phagemid vector pBluescript (Stratagene) and prior to commencement of all of the mutagenesis reactions except R47H, the insert was removed by digestion with *Bgl* II and recloned to obtain the reverse orientation of the insert in the vector. This enabled the coding region to be removed by both *Bgl* II and *Eco* RI, and rescue of the appropriate strand for annealing to the mutant oligonucleotides. Site-directed mutagenesis was carried out by the method of Kunkel (1985) using the oligonucleotides shown in Table 2. All mutations were confirmed by double-stranded sequencing using a Sequenase kit (USB).

Expression of JHE

For all of the mutants except K522R, the JHE cDNA sequences were removed from pBluescript by digestion with *Bgl* II and transferred to the *Bgl* II site of the baculovirus transfer vector pAcUW21 (Gift from Dr R. D. Possee, NERC, IVEM, Oxford, U.K.) and used to co-transfect cell line IPLB Sf21 of *Spodoptera frugiperda* with polyhedrin negative *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA as described by Bonning *et al.* (1992). The K522R mutant was removed from pBluescript by digestion with *Eco* RI and cloned into the *Eco* RI site immediately adjacent to the *Bgl* II site in pAcUW21 then co-transfected with AcNPV DNA. Recombinant polyhedron positive virus was purified to homogeneity by sequential plaque assays using standard assay procedures (Bonning *et al.*, 1992). Expression of JHE was carried out in spinner cultures using serum free Ex-Cell 400 medium (JRH Biosciences). Recombinant JHE viruses were added to cells at a multiplicity of infection of <0.1 and allowed to grow for 7–10 days at 27°C before harvesting of the secreted JHE from the cell media. Control non-mutant JHE had previously been expressed in a baculovirus system (Hammock *et al.*, 1990).

Wild type JHE and E332Q JHE were expressed using a modified pGEM5Zf(−) plasmid (pGT) containing a T7 RNA polymerase promoter and ribosome binding site placed ahead of an ATG start codon located within an *Nco* I restriction endonuclease site (CC*ATG*G). A *Nco* I restriction site was placed at the ATG start codon of JHE by mutagenesis, as described above, and the enzyme cloned into the modified vector (pGT). JHE expression was obtained by placing the plasmid in *E. coli* strain JM109(DE3) which contains a T7 RNA polymerase gene under control of a lac promoter, and adding IPTG at log phase. JHE activity was determined by directly assaying 100 μl of culture broth as described below. The E332Q mutant of JHE was made directly in the pGT vector using the methods described above for JHE in pBluescript.

Analysis of expressed JHE protein

To confirm the baculovirus expression of JHE, concentrated cell media (Amicon-30 micro-concentrators) from each mutant were compared to expressed wild type JHE by SDS–polyacrylamide gel electrophoresis, native polyacrylamide gel electrophoresis (Fig. 1), narrow range (pH 4–6.5) iso-electric focusing (Pharmacia-LKB), and Western blotting of an SDS–PAGE gel with polyclonal rabbit antiserum raised against JHE (Fig. 2).

Measurement of JHE activity

The activity of the mutant and wild type JHE was determined using two different assays. The first assay was a partition assay as described by Hammock and Sparks (1977) using [^{3}H]JHIII as a substrate. No data were used which resulted from experiments outside of conditions giving a linear dependence upon both time and protein concentration and all assays were performed in triplicate with each assay in a triplicate routinely producing hydrolysis levels within 10% of the average % hydrolysis.

In addition, a spectrophotometric substrate was used which allowed colorimetric detection of juvenile hormone esterase based upon the hydrolysis of a thioester. The substrate used is described by McCutchen *et al.* (Manuscript in preparation; see Table 3). Enzyme samples were diluted in reagent buffer containing 0.01% BSA, 0.015% DTNB [5′5′-dithio-bis-(2-nitrobenzoic acid)] in sodium phosphate buffer pH 7.4 and aliquoted to individual wells of a 96 well microtitre plate in triplicate. Each substrate was added to a final concentration of 5×10^{-4} M and the resulting reaction monitored in a continuous fashion on a Molecular Devices Corp. $V_{max}^{®}$ reader at 405 nm.

Determination of kinetic parameters

For the determination of the kinetic parameters for wild type JHE and the mutants containing activity (R47H, K29R and K522R), the enzymes were purified by DEAE chromatography as described by Ichinose *et al.* (1992). Briefly, cell free medium with catalytic activity was diluted 4-fold with distilled water and the pH adjusted to 8.5 with Tris–HCl at a final concentration of 2.5 mM. The diluted samples were applied to DEAE–Sepharose equilibrated in 10 mM Tris–HCl pH 8.5 containing 50 mM NaCl and eluted in the same buffer using a gradient of 50–200 mM NaCl. The JHE containing fractions were determined using the radiometric assay described above and concentrated using Centricon-30 microconcentrators (Amicon). The samples were equilibrated in 0.05M sodium phosphate buffer during concentration. The K_m, V_{max} and k_{cat} were determined using both substrates described above. Cell media and BSA were run as controls against both substrates. All assays were performed in triplicate.

Sensitivity of radiometric assay

To determine how much less active the catalytically inactive mutants of JHE are relative to wild type JHE it was necessary to improve the sensitivity of the assay by lowering the substrate concentration to 1×10^{-8} M. Since this substrate concentration does not exceed the K_m for JHE, the resulting data are not directly comparable to those expressed in Table 3. Rather these data give an estimate of relative enzyme concentration or activity. A series of dilutions of wild type JHE in sodium phosphate buffer pH 7.4 containing 0.01% BSA were assayed against JHIII in 30 min assays containing either 5×10^{-6} M JHIII or 1×10^{-8} M JHIII. In addition, to investigate if we could further improve the sensitivity of the assay by increasing the incubation time, some of the JHE dilutions for the low substrate concentration experiments were incubated for 1000 min (16 hr, 40 min) in 50 mM sodium phosphate buffer pH 7.4 containing 0.2% sodium azide, at 30°C, before being assayed. This enabled us to determine if JHE was sufficiently stable to allow a reliable 1000 min assay or if JHE would break down during that period and thus not give valid results.

pH profile

To determine if JHE was stable at high pH, wild type JHE from cell media was diluted 100-fold in water containing 100 μg/ml BSA as a carrier protein, then 5 μl added to 95 μl of 50 mM buffer solution before use in the radiometric assay described above. Phosphate buffer was used for the pH range 6–8, Tris–HCl for pH 7.4–9 and Glycine–NaOH for pH 9–10.

RESULTS

Mutagenesis of JHE

Amino acids to be mutated were selected based upon conservation of sequences between JHE and other esterases (Table 1). The potential catalytic site mutations R47H, D173N, S201G and H446K were confirmed by sequencing with no additional changes being included in the mutant oligonucleotides (Table 2). The E332Q mutation and the two control mutations, K29R and K522R, were identified by the inclusion of a *Sac*I, *Hind*III, *Bgl*II sites respectively in the oligonucleotides used for mutagenesis. The inclusion of these sites changed the codon usage, but retained the same amino acid sequence at the position of the engineered restriction endonuclease site (Table 2). The presence of all mutations was confirmed by sequencing.

Expression of JHE

The presence of recombinant baculoviruses potentially capable of expressing JHE was determined by the presence of polyhedra within co-transfected Sf21 cells. In all of the mutants examined, polyhedra were observed in some cells after co-transfection and plaque assay. These plaques were purified by sequential plaque purification until no non-recombinant polyhedra negative virus had been observed by plaque assay for two successive assays. For wild-type JHE and mutants K29R and K522R, JH specific esterase activity was observed in the cell media, indicating that JHE was being successfully produced and secreted to the medium. To confirm that JHE was being expressed from the recombinant catalytic mutants, cell media was concentrated 25-fold on Amicon-30 micro-concentrators and 5 μl of the concentrates were electrophoresed on both native and SDS–polyacrylamide gels and compared to expressed wild-type, K29R and K522R JHE which had been purified by DEAE chromatography. The major protein produced in all samples except K522R co-migrated with BSA on SDS–PAGE, which matches

Table 1. Comparison of some conserved motifs of esterases and lipases

Enzyme (ref)	$R_{47}F$ motif	$D_{173}Q$ motif	$G \times S_{201} \times G$ motif	$H_{446}G \times D/E$ motif
H. virescens JHE (C)	P V G E L R F K E L E P	P G N A G L R D Q V T L L R W V	T I A G Q S A G A	H E G V G H I E D L T Y V F
Culex Esterase (G)	P E G E L R F K A P V P	P G N A G L K D Q N L A I R W V	T L A G H S A G A	L R G T A H A D E L S Y L F
D. melanogaster E6 (B)	P T G D L R F E A P E P	P G N Y G L K D Q R L A L K W I	L L V G H S A G G	D F G T V H G D D Y F L I F
D. melanogaster AChE (C)	P V E D L R F R K P V P	P G N V G L W D Q A L A I R W L	T L F G E S A G G	W M G V L H G D E I E Y F F
Mouse AChE (H)	P V G S R R F M P P E P	P G N V G L L D Q R L A L Q W V	T L F G E S A G A	W M G V P H G Y E I E F I F
Bovine AChE (H)	P V G V R R F L P P E P	P G N V G L L D Q R L A L Q S V	T L F G E S A G A	W M G V P H G Y E I E F I F
Human AChE (H)	P L G F L R F K K P Q S	P G N M G L F D Q Q L A L Q W V	T L F G E S A G A	W M G V M H G Y E I E F V F
Torpedo AChE (C)	P V G N M R F R R P E P	P G N V G L L D Q R M A L Q W V	T I F G E S A G G	W M G V I H G Y E I E F V F
TCDD Esterase (F)	P L G S L R F A P P Q P	A V N R W V Q D N I A N F G G D	T I P G E S A G G	T V I G D H G D E I F
Dictystelium Est. CP (A)	–	S G N F G F L D Q V M A L D W V	T I Y G E S A G A	E G L V C H G T E L P M V F
Dictystelium EST. D2 (A)	–	H G N Y G F I D Q I K A L E W V	T I W G E S A G A	D D K V C H G T E L S L F F
Geotrichum GC Lipase (I)	P L N D L R F K H P Q P	N T N A G L K D Q R K G L E W V	M I F G E S A G A	F L G T F H G N E L I F N F
Geotrichum CC Lipase (I)	P V G N L R F K D P V P	S A N A G L K D Q R L G M Q W V	T I F G E S A G S	V L G T F H S N D I V F Q D
Human Panc. Lipase (J)	T N R K T R F I I H G F	G R I T G L - D P A E P C F Q C	H V I G H S L G A	F A A C N H L R S Y K Y Y T
Lysophospholipase (D)	–	P G N F G L R D Q H M A I A W V	T I F G E S A G G	W M G A D H A D D L Q Y V F
Milk Lipase (E)	–	P G N Y G L R D Q H M A I A W V	T L F G E S A G G	W V G A D H A D D I Q Y V F

The highly conserved motifs are underlined and the absence of a motif in an enzyme is indicated by a dashed line. For all of the esterases, the arginine residue (R) in the RF motifs occurs between positions 41 and 47 except for *Drosophila melanogaster* acetylcholinesterase (DmAChE) for which the arginine residue is at position 81. For the lipases, the arginine occurs at position 37 or 38. The aspartate residue (D) in the DQ motifs occurs between positions 160 and 190 for all enzymes except DmAChE (position 245). The serine residue (S) in the G × S × G motifs occurs between positions 188 and 217 for all enzymes except DmAChE (position 276) and Human Pancreatic Lipase (HPL; position 152). The histidine (H) in the HGxD/E motifs occurs between residues 435 and 447 for all enzymes except DmAChE (position 518) and HPL (position 263). The source references for these sequences in alphabetical order are: (A) Bomblies *et al.* (1990); (B) Cooke and Oakeshot (1989); (C) Hanzlik *et al.* (1989); (D) Han *et al.* (1987); (E) Hui *et al.* (1990); (F) Korza and Ozols (1988); (G) Mouches *et al.* (1990); (H) Rachinsky *et al.* (1990); (I) Schrag *et al.* (1991); (J) Winkler *et al.* (1990).

Table 2. Oligonucleotides used for the mutagenesis of JHE

R47H	JHE sequence 5′	AAG	CAG	CCT	GTT	GGA	GAA	CTC	Arg **AGG**	TTT	AAG	GAG	CTC	GAG	CCT
	Oligonucleotide 5′	AAG	CAG	CCT	GTT	GGA	GAA	CTC	His **CAC**	TTT	AAG	GAG	CTC	GAG	CCT
D173N	JHE sequence 5′	GCC	GGT	CTC	CGG	Asp **GAT**	CAG	GTA	ACC	CTG					
	Oligonucleotide 3′	CGG	CCA	GAG	GCC	TTA	GTC	CAT	TGG	GAC					
S201G	JHE sequence 5′		ATA	GCG	GGG	Asn CAG	Ser **AGC**	GCT	GGT	GCA	TCA				
	Oligonucleotide 3′		TAT	CGC	CCC	GTC	CCG	CGA	CCA	CGT	AGT				
E332Q	JHE sequence 5′	ATA	GGA	TTT	ACC	AGC	Gly TCA	Glu **GAA**	TGC	GAG	ACT				
	Oligonucleotide 3′	TAT	CCT	AAA	TGC	TCG	AGT	GTT	ACG	CTC	TGA				
H446K	JHE sequence 5′	GAG	GGT	GTC	GGC	His **CAC**	ATT	Gln GAG	GAC	TTA					
	Oligonucleotide 3′	CTC	CCA	CAG	CCG	TTC	TAA	CTC	CTG	AAT					
K29R	JHE seqence 5′		GAT	GGC	ATC	Lys AAG	Lys **TTC**	GCC	AGC	TTC					
	Oligonucleotide 3′		CTA	CCG	TAG	TCC	AAC	CGT	TCG	AAG					
K522R	JHE sequence 5′	ATC	ATC	AGA	TCC	Lys **AAG**	Arg GAG	TTC	GCC						
	Oligonucleotide 3′	TAG	TAG	TCT	AGA	GCC	CTC	AAG	CGG						

The original amino acid is shown above the sequence for each mutation and the subsequent change is shown below the sequence. The altered nucleic acids are shown in bold. Restriction endonuclease sites incorporated for screening purposes are underlined.

the expected size of 66 kDa for expressed JHE (Hammock *et al.*, 1990), and no difference was discernible between mutant and non-mutant JHE on either SDS or native gels (Fig. 1). The K522R protein did not express to the same levels as the other samples, but was still obviously produced. No equivalent 66 kDa protein was observed in uninfected cell media and Western blotting of an SDS–PAGE gel with anti-JHE antiserum confirmed the 66 kDa bands as JHE (Fig. 2).

To confirm that the mutations had not caused gross conformational or charge changes upon JHE, JHE was analyzed by narrow range (pH 4.0–6.5) iso-electric focusing using a Pharmacia-LKB slab system. Analysis of eluted gel slices for wild type JHE confirmed the major protein band as having JH esterase activity with a pI of 4.9 as expected for the JHE of *H. virescens* (Fig. 2) (Abdel-Aal *et al.*, 1988). No differences were discernable among mutant and wild-type JHE's by migration (data not shown). Determination of protein concentrations by densitometry with comparison to known BSA concentrations indicated JHE was expressed in the cell media at greater than 10 μg/ml for wild-type and mutant forms of the enzyme.

Analysis of esterase activity of mutant and non-mutant JHE

Comparison of the activity of DEAE purified wild type, K29R and K522R JHE showed no major differences with either substrate examined (Table 3). However, analysis of mutants R47H, D173N, S201G and H446K, showed a dramatic drop in activity for R47H and no detectable activity for the Ser, His or Asp mutants.

As controls, the activity of Sf21 and cell media, and BSA were determined with both substrates. No activity was detectable with either of the JH substrates (data not shown).

To determine if levels of activity below our detection limits were present, we developed an assay procedure with increased sensitivity. [^{3}H]JHIII substrate was used at a limiting concentration of 1×10^{-8} M, and the assay time was increased to 1000 min. This assay was used in an attempt to detect esterase activity for the Ser, His and Asp mutants. Although not comparable with the standard assay run at 5×10^{-6} M, the results presented in Fig. 3 show that even at JHIII concentrations as low as 1×10^{-8} M, the hydrolysis of JH by wild type enzyme is linearly dependent upon protein concentration up to 50% hydrolysis of the substrate. The sensitivity of the 30 min assay shown in Fig. 3, as determined by % hydrolysis, is approx 30-fold greater at 1×10^{-8} M than at 5×10^{-6} M JHIII (1650 pg vs 53 pg for 30% hydrolysis in 30 min). Figure 3 also shows that incubation of wild-type JHE for 1000 min at 30°C before use in the 30 min assay does not inactivate the enzyme. The conjugated methyl ester of JH is also stable for such long incubation times (data not shown). These data imply that it is possible to run an assay for low levels of JHE activity using 1×10^{-8} M JHIII and a 1000 min assay.

Even the increased sensitivity obtained by using very low substrate concentrations and 1000 min assays did not enable us to detect any JHE activity for the Ser, His and Asp mutants. As a low substrate concentration assay is approx 30-fold more sensitive than the higher concentration assay; the 1000 min assay should be 33-fold more sensitive than a 30 min assay;

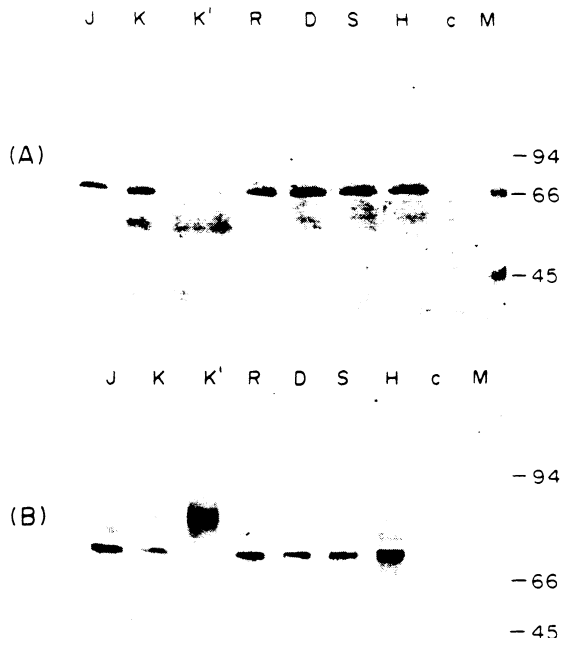

Fig. 1. SDS (A) and Native (B) polyacrylamide gel electrophoresis of wild type and mutant JHE's. (J) wild type JHE; (K) K29R; (K') K522R; (R) R47H; (D) D173N; (S) S201G; (H) H446K; (c) cleared cell media; (M) markers. J, K and K' samples were purified by DEAE chromatography and all other samples were concentrated directly from cell media prior to electrophoresis on 4–15% precast gradient gels (BioRad)

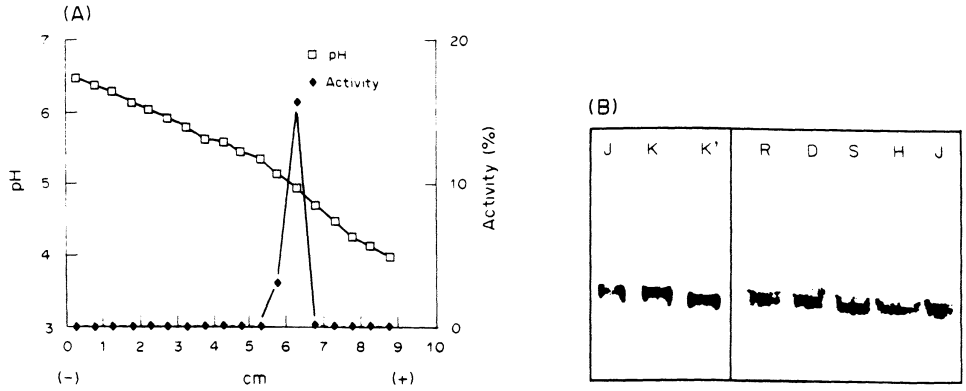

Fig. 2. Iso-electric focusing (A) and Western blot analysis (B) of wild type and mutant JHE's. Electrofocusing was performed on a horizontal slab gel with a pH 4.0–6.5 gradient for 2 hr at 4 C. Following electrofocusing the JHE lane was sliced into 5 mm segments and eluted overnight with 50 mM sodium phosphate buffer pH 7.4 for enzyme activity (◆) or 20 mM KCl for pH determination (□). Staining of the gel indicated that all mutant JHE's yielded a band with a p*I* indistinguishable from wild type enzyme. Western blot analysis was carried out by electrophoresis of JHE's on a 10% SDS–PAGE gel followed by semi-dry transfer to nitrocellulose. The blot was probed with rabbit antiserum to JHE and the probe detected with alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma) using NBT BCIP substrate. J, K, K′ samples were analyzed on a separate gel to the remaining samples. (J) wild type JHE; (K) K29R; (K′) K522R; (R) R47H; (D) D173N; (S) S201G; (H) H446K.

and the Asp, Ser and His mutants were not diluted 1000-fold before use as occurred for the wild-type JHE; the implication is that these mutants are at least 10^6-fold less active than wild-type JHE. No activity was detectable for the E332Q mutant of JHE when expressed in the *E. coli* system and this mutant was found to be at least 100-fold less active than wild type JHE. Due to the poor expression levels in *E. coli* it was not possible to determine if this mutant is greater than 100-fold less active than wild type JHE.

Kinetic parameters

Analysis of the wild type JHE and catalytically active mutants of JHE, indicated that the K29R and K522R mutants possessed very similar K_m, k_{cat} and V_{max} parameters to the wild type enzyme (Table 3). The R47H mutant was found to have a K_m of 0.066 μM and a k_{cat} of 1.9 min^{-1} and is approx 50-fold less active than the wild type JHE.

Table 3. Kinetic parameters for wild-type and mutant JHE

Substrate	Enzyme	K_m (μM)	V_{max} (μmol/min/mg)	k_{cat} (min^{-1})
JHIII				
	Wild-type	0.03 ± 0.01	1.57 ± 0.06	104
	R47H	0.07 ± 0.02	0.03 ± 0.002	1.90
	K29R	0.05 ± 0.01	1.89 ± 0.05	125
	K522R	0.06 ± 0.01	3.00 ± 0.21	198
	D173N		No detectable activity	
	S201G		No detectable activity	
	H446K		No detectable activity	
	E332Q		No detectable activity	
$C_6H_{13}OCH_2C(O)SCH_3$				
	Wild-type	46 ± 6	55 ± 3	3649
	R47H	20 ± 6	0.6 ± 0.1	37.6
	K29R	49 ± 7	67 ± 4	4466
	K522R	77 ± 19	118 ± 14	7800
	D173N		No detectable activity	
	S201G		No detectable activity	
	H446K		No detectable activity	
	E332Q		No detectable activity	

Kinetic parameters were determined using the assay procedures described in the Materials and Methods with [^{3}H]JHIII and $C_6H_{13}OCH_2C(O)SCH_3$ as substrates. For the radiometric assay, JHIII substrate concentrations were 20, 40, 80, 160, 320 and 640 nM with assay times and JHE concentrations adjusted to keep the substrate hydrolysis below 30%, which is within the linear range for this enzyme (see Fig. 3). For the spectrophotometric substrate, the kinetic parameters were determined directly using a Molecular Devices Corp. V_{max} reader. All enzyme dilutions were carried out in 0.05 M sodium phosphate buffer containing 10% sucrose and 0.01% BSA as carriers. All catalytically active proteins were purified by DEAE chromatography, as described in Materials and Methods.

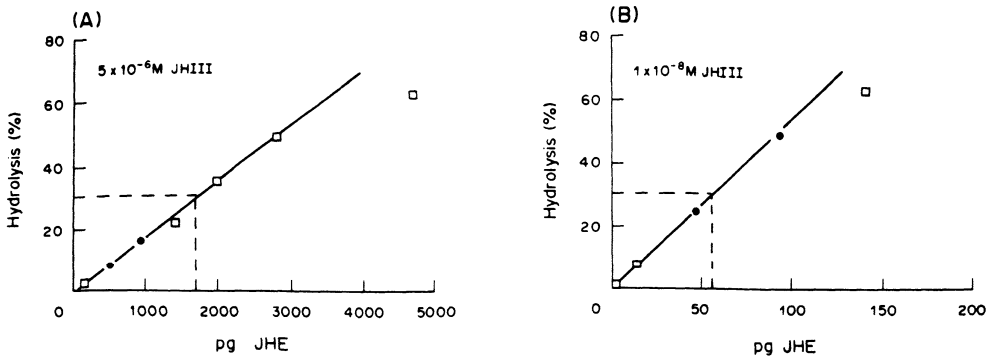

Fig. 3. Effect of JHIII substrate concentration upon detection of JHE. Analysis of picograms of JHE in a 100 μl assay vs percent hydrolysis after a 30 min incubation at 30°C using JHIII at a concentration of 5×10^{-6} M (A) or 10^{-8} M (B). (□) Represents direct assay of JHE and (●) indicate preincubation of the sample at 30°C for 1000 min prior to addition of racemic JHIII and commencement of the 30 min assay. The dashed line represents the amount of JHE required to hydrolyze 30% of the substrate in 30 min at 30°C. All data were within 10% of the average % hydrolysis presented here.

pH stability

Studies of chymotrypsin (Sprang *et al.*, 1987; Craik *et al.*, 1987) have indicated that inactivation of this enzyme by mutagenesis of the catalytic Asp to Asn can be partially reversed by increasing the pH of the assay to greater than 10. To determine if this was possible for JHE and thus have evidence of a catalytic role for Asp_{173}, we performed a pH study on wild type JHE and D173N JHE. Analysis of the pH profile (Fig. 4) indicates that the pH optimum for wild type JHE is between 7 and 9, and that by pH 10, the JHE is mostly inactive. This result indicates that attempting to obtain activity for the D173N mutant at high pH is unlikely to work even if the mutation is catalytic rather than structural. This lack of activity at high pH was confirmed for the D173N mutant (data not shown).

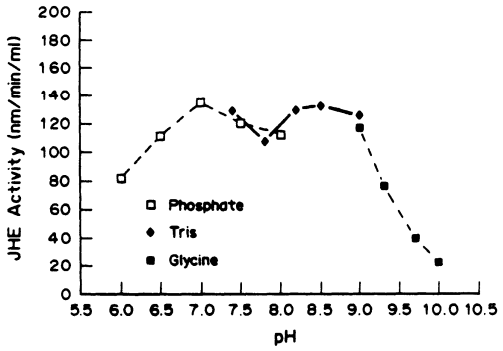

Fig. 4. pH profile of JHE activity. A standard 100 μl JHE assay was performed as described in Materials and Methods. Phosphate represents 50 mM sodium phosphate buffer (pH 6–8). Tris represents 50 mM Tris–HCl buffer (pH 7.4–9). Glycine represents 50 mM Glycine–NaOH buffer (pH 9–10). All points were assayed in triplicate with the range being less than 10% of the value shown for all points.

DISCUSSION

The mutagenesis studies carried out in this report have highlighted a number of residues important to the catalytic activity of JHE. The results obtained from the S201G, H446K and D173N mutations would indicate that a Ser–His–Asp catalytic triad is critical to the mechanism of JH hydrolysis by this enzyme. However, one of the major problems associated with the use of site-directed mutagenesis in isolation is that the change of an individual amino acid may influence catalysis indirectly by changing structure, therefore, it is possible that the amino acids changed are essential for structural integrity but are not involved directly in catalysis. In order to definitively identify the catalytic residues in any enzyme, a combination of site-directed mutagenesis, active site labelling, and crystal structure is ideal. This paper presents the site-directed mutagenesis component of this research.

Analysis of the Ser residue at position 201 in JHE shows that it is represented within a consensus catalytic motif of G x S x G which is common to serine proteases, esterases and lipases (Brenner, 1988). Glycine was chosen to replace Ser at this position as Gly cannot function as a catalytic residue and should severely affect the action of JHE without grossly affecting structure. As we could not detect esterase activity for the S201G mutant of JHE and this Ser is within the consensus motif for an active site Ser, we believe this to be the active site Ser of JHE.

The His residue at position 446 in JHE is also located within a consensus motif (Table 1) and the equivalent His residue has been shown by site-directed mutagenesis to be the active site residue for pancreatic cholesterol lipase (DiPersio *et al.*, 1991) and acetylcholinesterase from *T. californica* (Gibney *et al.*, 1990). In addition, Korza and Ozols (1988) have shown His_{441} of the esterase induced by 2,3,7,8-tetrachloridobenzo-*p*-dioxin in rabbit liver to be in

the active site by amino acid sequencing of a peptide labelled by diisopropyl fluorophosphate. Mutation of His to Lys is a relatively conservative change as proposed for the Ser mutation, it is unlikely that the structural change would be sufficient to remove all detectable activity unless this were a catalytically active residue.

Additional evidence supporting the Ser_{201} and His_{446} residues being catalytically active in JHE comes from analogy to the structure of lipases from *Geotrichum candidum* (Schrag *et al.*, 1991) and acetylcholinesterase from *T. californica* (Sussman *et al.*, 1991). These enzymes show a large degree of homology to JHE and other esterases with the active site containing Ser_{217} and His_{463} for the lipases and Ser_{200} and His_{400} for the esterase.

Analysis of the highly conserved Asp_{173} residue indicated that this residue is essential for enzyme activity as we were unable to detect activity from the D173N mutant even at a low substrate concentration with long incubation times. This would appear to indicate that JHE possesses a Ser–His–Asp catalytic triad, however, the publications of Schrag *et al.* (1991) and Sussman *et al.* (1991) indicate that a glutamate residue is the third member of a charge relay system in some esterases and lipases. Our preliminary data for the E332Q mutant of JHE indicates that this Glu residue is important for enzyme activity, and based on the above studies, this would appear to be the most likely candidate for the third member of a catalytic triad. However, the lack of activity in the D173N mutant is also intriguing and it is possible that this residue plays an important role in the functioning of JHE.

To further determine the possible role of Asp_{173} in JHE we investigated the possibility of recovering some of the activity of this mutant by increasing the pH of the partition assay, as has been shown for the equivalent mutation in trypsin (Craik *et al.*, 1987). We were unable to recover activity by increasing the pH of the assay to 10, however, this can probably be explained by the progressive loss of activity in wild type JHE as the assay pH increases over 9.0. Despite the structural evidence presented by Schrag *et al.* (1991), not all lipases have a glutamate residue as the carboxylate member of a catalytic triad. Human pancreatic lipase (HPL) has been shown by structural analysis to have an aspartate residue at position 176 as the carboxylate residue in its catalytic triad (Winkler *et al.*, 1990) and as indicated in Table 1, this residue occurs in a conserved region of esterases and lipases.

Our experimental evidence indicates the important roles of both Asp_{73} and Glu_{332} in the functioning of JHE and the identified role of glutamate in the only esterase for which the crystal structure is known, points strongly to this being the catalytic residue in JHE, however, the possibility of Asp_{173} being involved and its obvious importance means this residue can not be ignored.

Hanzlik *et al.* (1989) proposed that the arginine residue at position 47 corresponded linearly with the active site His_{57} in chymotrypsin and might either function as an alternative to His for the hydrolysis of esters, or act to orient water for successful catalysis of substrates. Mutation of Arg_{47} to His caused the activity of JHE to drop approx 50-fold. From this, and the apparently important role of His_{446}, we feel it unlikely that this residue is intimately involved in a catalytic triad *per se* although it obviously plays an important role. It is possible that Arg47 does orient water within the catalytic site, thus improving the efficiency of hydrolysis, without actual involvement in the catalytic triad. Alternatively, it may be more appropriate to consider Arg_{47} as a fourth member of a catalytic quadrad. Other possibilities for the role of Arg_{47} include involvement in the binding of JH substrates or in enzyme structure. We have no evidence for either of these possibilities other than conservation in many esterases and the apparently important role Arg_{47} plays in the functioning of JHE.

In summary, our study supports the hypothesis that Ser_{201} and His_{446} are in the catalytic triad of JHE and, by sequence comparison, in other esterases and lipases. The Arg_{47} residue studied here appears to be important for the activity of JHE but may not be part of a catalytic triad and is unlikely to be an alternative to histidine during substrate hydrolysis. A more likely role for Arg_{47} is in the orientation of water in the active site, thus improving the catalytic efficiency of JHE.

Analysis of Asp_{173} and Glu_{332} indicated that both of these residues are important for JHE activity. From site-directed mutagenesis alone it is not possible to definitively distinguish between the importance of an amino acid for structure versus a direct catalytic role, and further research in the form of structure determination and active site labelling is required to determine if JHE is similar to acetylcholinesterase with a catalytic Glu residue at position 332, or if JHE more closely resembles human pancreatic lipase with a catalytic aspartate residue at position 173. What these studies have told us, is that there is an absolute requirement for both Asp_{173} and Glu_{332} if JHE is to function effectively.

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