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STUDIES OF SPECIFICITY AND INHIBITION OF HUMAN CEREBROSPINAL FLUID DYNORPHIN CONVERTING ENZYME

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Dynorphin-converting activity was recently discovered in human cerebrospinal fluid.[1] This enzyme (hCSF-DCE) cleaves dynorphin A, dynorphin B and alpha-neoendorphin to release Leu-enkephalin-Arg[6]. To characterize the enzyme further we used several protease inhibitors, including *N*-peptidyl-*O*-acyl hydroxylamines which are known to act as potent irreversible inhibitors of serine and cysteine proteinases.[2-4]

No irreversible inactivation occurred but strong, reversible effects on the dynorphin-converting activity by some of the inhibitors tested could be observed. Although, hCSF-DCE binds its substrates (dynorphin A and B) in the μM-mM concentration range, it exhibits high specificity in recognizing and cleaving the linkage between the two basic amino acids in the substrate sequence.

KEY WORDS: Opioid peptides, dynorphin degradation, proteinase inhibitors, serine protease.

INTRODUCTION

Limited proteolysis is crucial in co- and posttranslational modification of peptides and proteins causing either sequential processing to biological active peptides or their final degradation to inactive products. Cellular systems make use of proteolytic peptide processing to balance chain-length dependent conformational states of peptides exhibiting different biological activities (e.g. binding differences to several types of receptors).[5] Depending on the regulatory process, the function of the peptide in the particular tissue, the state of compartmentalization, several proteinases (or types of proteinases) may be involved in the control and the termination of action of bioactive peptides. For instance, the kappa-receptor active opioid neuropeptides dynorphin A and dynorphin B are released from prepropetides as prodynorphin and leumorphin.[6,7] Their further processing by serine and cystein proteinases generate the delta-receptor active opioids Leu-enkephalin and Leu-enkephalin-Arg[6].[1,8,9] Their action finally may be terminated by membrane endo- and exopeptidases.[10] Imbalance of such regulatory

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ABBREVIATIONS: hCSF-DCE, human cerebrospinal fluid – dynorphin converting enzyme; HPLC, high performance liquid chromatography; RIA, radioimmunoassay; TRIS, tris(hydroxymethyl) aminomethane; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethylsulfoxide; Boc-, tert. butyloxycarbonyl; Z-, benzyloxycarbonyl; -pNA, 4-nitroanilide; -Nb, 4-nitrobenzoyl; -Bz, benzoyl; -Ma, methacryl; Suc-, succinyl.

For discussion of proteinase-peptide interactions the nomenclature of Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.*, **27**, 156, has been used.

cascade may be related to numerous physiological malfunctions.[11] Therefore the study of those peptide hormone processing enzymes may lead to new effectors useful in the treatment of disorder.[12,13]

We have investigated the activity of hCSF-DCE aginst synthetic chromogenic substrates and its reaction with peptide-derived inhibitors to increase our knowledge on the specificity of the enzyme.

MATERIAL AND METHODS

Enzyme

Dynorphin-converting-enzyme was purified from human cerebrospinal fluid as described by Nyberg *et al.*[1] Final purification of the active fractions collected after molecular sieving at Sephadex G-100 has been performed by HPLC on a TSK G-3000 SW column (see Figure 1). The active fractions were collected, desalted on Sephadex G-10 and freeze-dried. The protein concentration of solutions is based on the weight.

Substrates and Inhibitors

Suc-Ala_3-pNA, Z-Arg-pNA and PMSF were from Sigma. Suc-Ala-Ala-Pro-Phe-pNA was from Bachem. Suc-Ala_2-Phe-pNA, Z-Ala_2-Phe-CH_3 and Z-Ala_2-Phe-CH_2Cl were gifts of Dr. S. Fittkau, Department of Biochemistry, University of Halle, G.D.R. H-Ala-pNA*HCl, H-Ala_2-pNA*HCl, H-Ala_3-pNA*HCl, H-Gly-Pro-pNA*HCl and

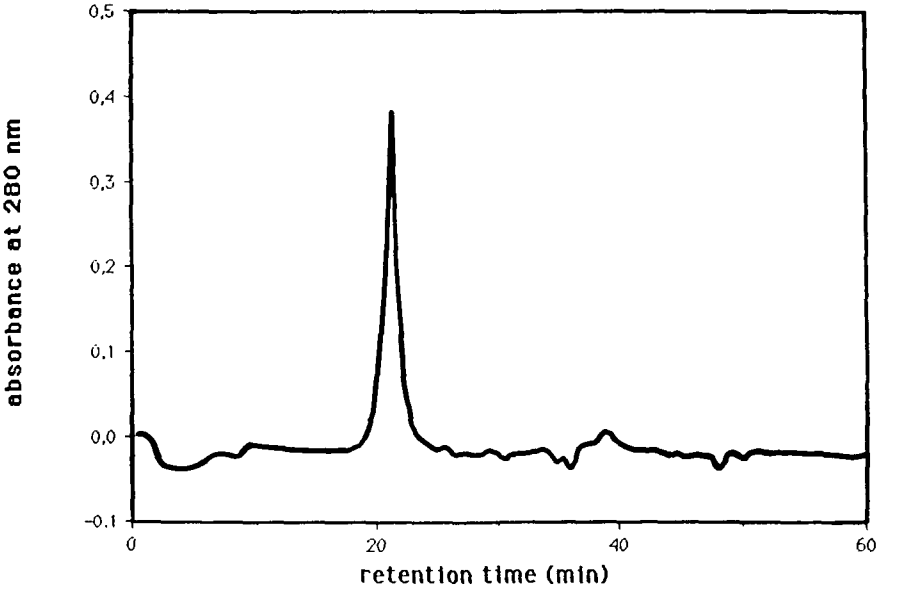

FIGURE 1 Elution profile of hCSF-DCE after HPLC-gel filtration The separation was performed on a 2140-HPLC-system (LKB, Bromma, Sweden) using a TSK G-3000 SW column and 20 mM Tris-HCl buffer, pH 7.8 as eluent.

Boc-Gly-Pro-pNA were gifts of Dr. K. Neubert, Department of Biotechnology, University of Halle, G.D.R. H-Lys-Ala-pNA*HCl and Glt-Leu-Phe-pNA were provided by Dr. U. Neumann, Department of Pharmacology, Medical Academy of Erfurt, G.D.R.

Dynorphin A was purchased from Bachem (Bubendorf, Switzerland), dynorphin B was prepared by Dr. G. Lindeberg, Department of Immunology, University of Uppsala (Sweden) and Leu-enkephalin-Arg^6 was from Peninsula Laboratories (Belmont, U.S.A.).

N-peptidyl-*O*-acyl hydroxylamines were synthesized as described by Fischer and Demuth.[4,14,15]

For the enzyme assay (see below) stock solutions of the inhibitors or chromogenic substrates were prepared in DMSO or methanol (4.0 mM) and diluted with distilled water to give final concentrations of 0.4 mM. Further dilution of those solutions were prepared in order to investigate the concentration dependence of the inhibition. In no case was the concentration of organic solvent higher than 2.5% (v/v).

Enzyme Inhibition and Activity

Preincubations of enzyme with different inhibitors were performed between 30 and 60 min (see Results and Discussion) at 37°C. Usually, volumes of 10 μl samples of the reagents solutions (buffer, enzyme, inhibitor or water) were pipetted.

The activity assay, after preincubation was finished, was initiated by addition of 10 μl dynorphin A or dynorphin B solutions. The typical assay volume of 40 μl contained 20 mM Tris-HCl buffer, pH 7.8, 1.5 μg enzyme and 1.2 μM dynorphin A (or 1.6 μM dynorphin B) as substrate. The incubations were performed in Eppendorf tubes at 37°C. For the kinetic studies aliquots of the enzyme assay mixtures have been diluted up to 100 times before applying the samples to the following RIA-procedure. The reaction was terminated by the addition of 0.5 ml of ice-cold methanol followed by centrifugation and evaporation of the supernatant.

The enzyme activity was monitored by measuring the formation of Leu-enkephalin-Arg^6 in the tubes from synthetic dynorphin A or dynorphin B using radioimmunoassay (RIA) specific for the product. This assay was based on the charcoal adsorption technique and was conducted as described previously.[19,20]

Additionally, the activity of hCSF-DCE against several chromogenic peptide substrate was analyzed at 405 nm using a Titertek Multiscan MC filter photometer. Incubations were made in Eppendorf tubes at 37°C using 110μl sample volume, containing 20 mM Tris-HCl buffer, pH 7.8, 3μg enzyme per assay and concentrations of the chromogenic substrates of 0.46 mM. After 60 h reaction time at 37°C, the reaction mixtures were transferred to microtitration plates and the release of 4-nitroanilide estimated using blanks containing all reagents besides the enzyme. An extensive description of this new method has been published elsewhere.[18]

Monitoring of the enzyme activity by HPLC chromatography was conducted as described essentially previously.[1,9] HCSF-DCE was preincubated for 4 h with five different inhibitors at 37°C. Then the enzyme-catalyzed release of Leu-enkephalin-Arg^6 was initiated by adding a solution of dynorphin A. The reaction was allowed to proceed for 2 h at 37°C before a 200 μl aliquot was removed and applied to a reversed phase HPLC-column, which was subsequently eluted using a water-acetonitrile gradient.

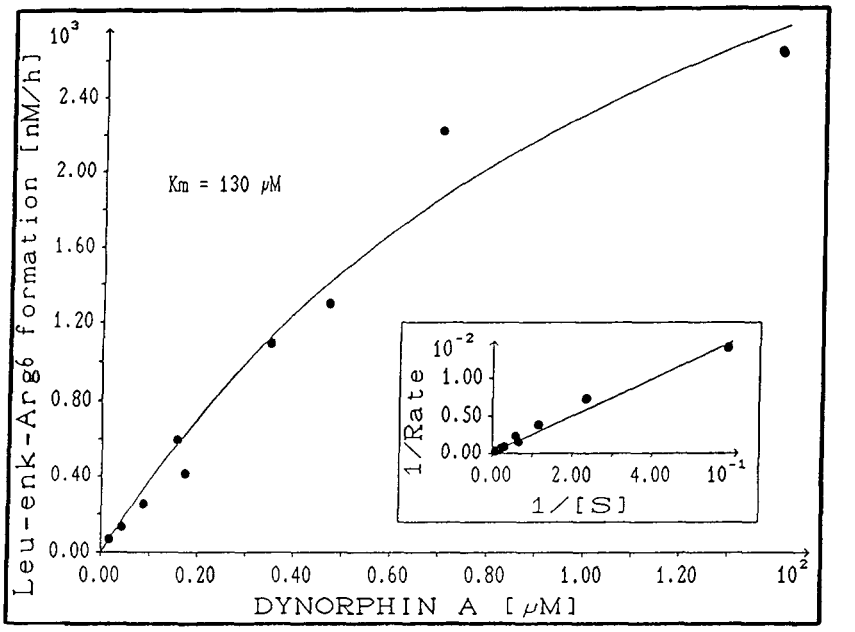

FIGURE 2 Dependence of the hCSF-DCE catalyzed release of Leu-enkephalin-Arg^6 from dynorphin A on the concentration of the substrate (inset: Lineweaver-Burk-Plot). hCSF-DCE was incubated with substrate solution in 20 mM Tris-HCl buffer, pH 7.8, 37°C. The typical incubation volume was 40 μl and the enzyme concentration 1.5 μg protein per assay. After 60 min incubation time the reaction was stopped by adding ice-cold methanol, the samples were diluted up to 100 times and the product formation assayed by RIA (see Material and Methods).

RESULTS AND DISCUSSION

Incubation of the specific substrates dynorphin A and dynorphin B with hCSF-DCE for 60 minutes results in the release of substantial amounts of Leu-enkephalin-Arg^6. Establishing initial rate conditions we estimated the kinetic parameters of these hydrolytic reactions (Figures 2 and 3). With a K_m value of 130 μM ($\pm$ 33%) and a specific activity of 2.4 mU/mg enzyme protein, dynorphin A is 6 times more rapidly hydrolyzed compared to dynorphin B (K_m = 160 μM $\pm$ 13%, specific activity = 0.4 mU/mg protein). Since the binding constants are almost identical for both substrates converted by hCSF-DCE to the same product, Leu-enkephalin-Arg^6, the acylation step during catalysis seems to be rate-determinng. The structural differences in the C-terminal region of both dynorphins (see table I) might be responsible for the observed difference in the specificity.

Since human CSF dynrophin-converting enzyme exhibits some properties of a serine protease[1] we tested its activity against chromogenic substrates known to be specific for enzymes of the trypsin family of serine proteases (i.e. chymotrypsin, elastase, trypsin). Even after 60 hours reaction time no hydrolysis could be detected.

Peptidyl methylketone derivatives and *N*-peptidyl-*O*-acyl hydroxylamines have been shown previously to be specific inhibitors of several serine and cysteine proteinases.[2-4,16,17] To characterize hCSF-DCE further we have studied the influence of

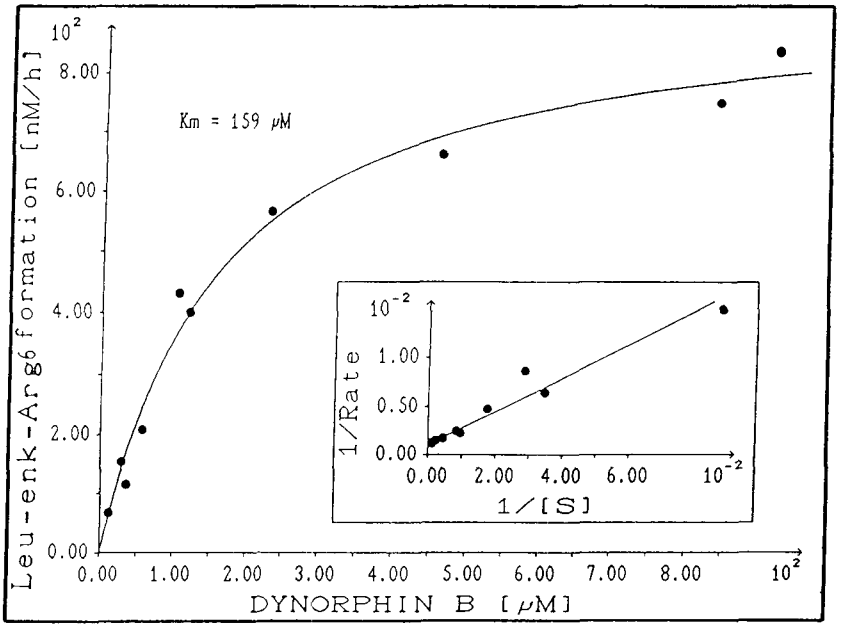

FIGURE 3 Dependence of the hCSF-DCE catalyzed release of Leu-enkephalin-Arg^6 from dynorphin B on the concentration of the substrate (inset: Lineweaver-Burk-Plot). The method followed is described under Figure 2.

these inhibitors on the activity of the enzyme using the radioimmunoassay method. Preincubation of the enzyme with the compounds resulted in substantial loss of the enzymic activity, as detected in the subsequent enzyme assay using both dynorphin A and B as substrates and the RIA-method for activity estimation (Table 1). Interes-

TABLE I
Effects of several peptide inhibitors on the activity of hCSF-DCE towards dynorphin A and dynorphin B after 30 min preincubation*

inhibitlor§	dynorphin A$^+$ activity	dynorphin B$^+$ activity
Boc-Ala-Ala-NHO-Nb	72.1	78.7#
Z-Phe-Phe-NHO-MA	17.7	1.0
Boc-Phe-Gly-NHO-Bz	75.6	39.1
Boc-Ala-Pro-Val-NHO-Nb	86.7	58.9
Z-Ala-Ala-Phe-CH_2-Cl	52.1	28.5
Z-Ala-Ala-Phe-CH_3	51.2	7.6
Boc-Ala-Ala-CH_2-Cl	80.2	55.9
PMSF	19.3	13.9

* 30 min preincubation at 37°C, 60 min enzyme assay with substrates at 37°C
# values as percentage of controls containing a maximum of 2.5% organic solvent, estimated by RIA (see Materials and Methods)
† substrate concentrations: dynorphin A (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln) = 1.2×10^{-6} μM, dynorphin B (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Glyn-Phe-Lys-Val-Val-Thr) = 1.6×10^{-6} μM
§ inhibitor concentrations = 0.1 mM

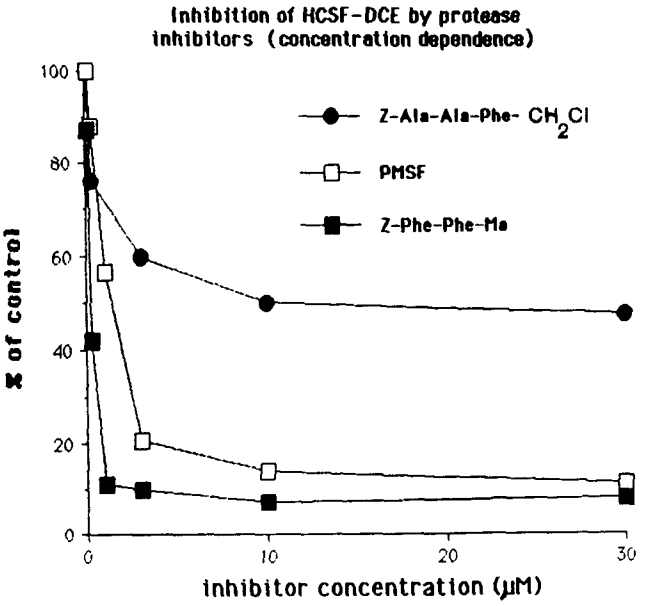

FIGURE 4 Concentration dependence of the inhibition of hCSF–DCE by protease inhibitors. After preincubation of hCSF-DCE with inhibitors at 37°C for 30 min the reaction mixture was incubated with substrate solution in 20 mM Tris-HCl1 buffer, pH 7.8, 37°C. The typical incubation volume was 40 μl, the enzyme concentration 1.5 μg protein per assay. After 60 min incubation time the reaction was stopped by adding ice-cold methanol, the product formation was assayed by RIA (see Material and Methods).

tingly, the compounds containing phenylalanine induced the strongest inhibition of hCSF-DCE. Investigating the influence of the inhibitor concentration on the inhibition of the target enzyme we found Z-Phe-Phe-NHO-Ma the most powerful inhibitor (Figure 4).

To confirm these results that have been evaluated using low substrate concen-

TABLE II
Residual activity of hCSF-DCE towards dynorphin A after 4 hours preincubation* of the enzyme with selected inhibitors

Inhibitors[$]	dynorphin A[+] activity (% of control)
Z-Phe-Phe-NHO-Ma	73.7[#]
Boc-Phe-Gly-NHO-Bz	71.4
Z-Ala-Ala-Phe-CH_2-Cl	100.0
Z-Ala-Ala-Phe-CH_3	105.0
PMSF	25.1

* 4 h precincubation of enzyme and inhibitors, followed by 60 min enzyme assay using dynorphin A as substrate; from these assays 200 μl samples have been withdrawn for HPLC-separation (See Figure 5); to estimate the amount of Leu-enkephalin-Arg[6] formed, aliquots of these assay mixtures were diluted 100-fold and subsequently analyzed by the RIA-technique

[#] values as percentage of controls estimated by RIA (see Materials and Methods)

[+] dynorphin A concentration for the HPLC enzyme assay was 56 μM

[$] inhibitor concentrations = 0.1 mM

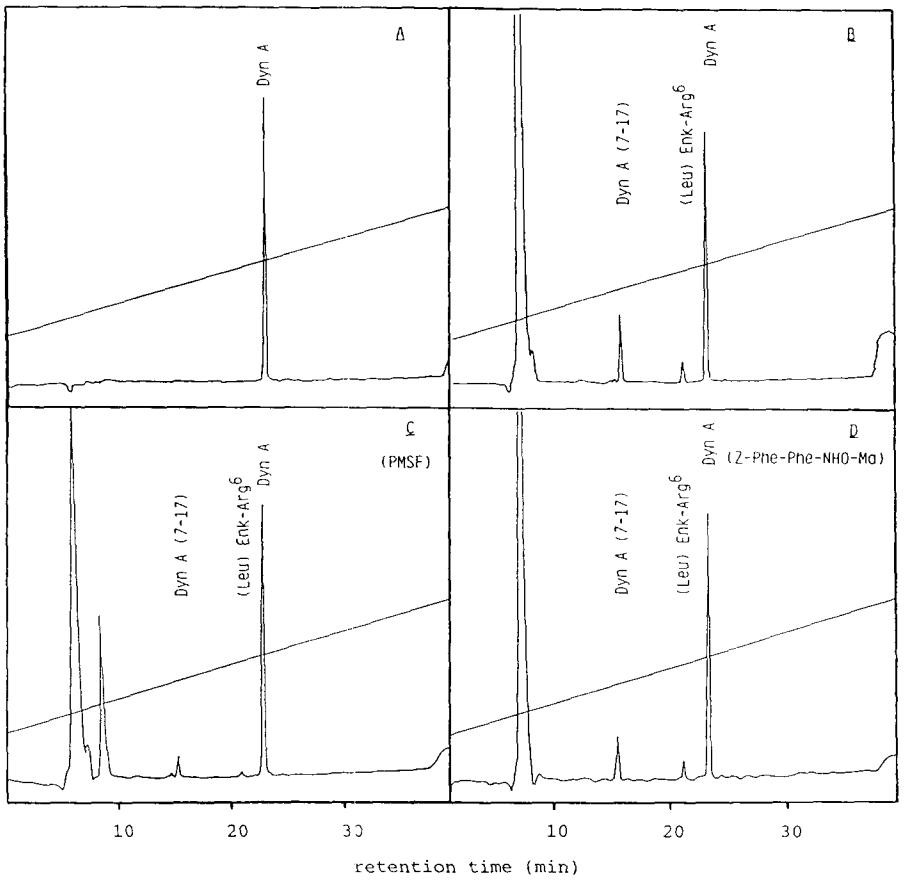

FIGURE 5 HPLC-elution profiles of the products formed during dynorphin A — degradation by hCSF-DCE preincubated with protease inhibitors. The enzyme was preincubated for 4 h at 37°C with 0.1 mM Boc-Phe-Gly-NHO-Bz, Z-Ala-Ala-Phe-CH_2-Cl, Z-Ala-Ala-Phe-CH_3 (chromatograms not shown, see Table II), with Z-Phe-Phe-NHO-Ma and PMSF. Subsequently, the enzyme solutions containing 30 μg protein per assay were incubated for 2 h at 37°C in 20 mM Tric-HCl buffer, pH 7.8, containing 56 μM dynorphin A. *A*: dynorphin A, *B*: dynorphin A and products of the hCSF-DCE catalyzed hydrlysis, *C*: same as *B*, but enzyme preincubated with Z-Phe-Phe-NHO-Ma. The ordinate in the chromatograms represents the absorbance followed at 210 nm from 0 up to 0.29 optical units.

trations (0.1 μg per assay tube corresponds to 1.2 μM/l of dynorphin A and to 1.6 μM/l of dynorphin B) we applied reversed phase HPLC, where up to 50 times higher substrate concentrations have been used (See Material and Methods). Figure 5 shows that only PMSF exhibits a strong effect on the hCSF-DCE activity. Samples of 10 μl of the incubates which had been prepared for the HPLC-analysis, were diluted 100-fold and assayed in parallel using the RIA-method as described in Material and Methods. The results listed in Table II show that preincubation of hCSF-DCE with peptide inhibitors which inactivate serine and cysteine proteinases effectively (i.e. Z-Phe-Phe-NHO-Ma and Boc-Phe-Gly-NHO-Bz), resulted in only a slight inhibition of the dynorphin converting enzyme after a prolonged preincubation time. Therefore,

the strong effects listed in Table I could be due to noncovalent interactions between the target enzyme and the peptide inhibitors in the presence of the lower substrate concentrations used for the RIA-enzyme assay.

From this study it is clear that hCSF-DCE is a highly specific enzyme cleaving selective substrates at a basic amino acid pair. It is not irreversibly inhibited by irreversible acting peptide inhibitors designed after other enzymes specificities. Its affinity for phenylalanine containing peptide structure may be due to the preference for hydrophobic interactions at the S′-binding site of the enzyme.

Work is in progress to elucidate whether hCSF-DCE can be classified as a member of the trypsin-family of serine proteases. Further studies will be directed towards the design of irreversible acting inhibitors for this enzyme.

Acknowledgement

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