EXTRACTION AND PURIFICATION OF DIAMINE OXIDASE FROM MARINE COPRODUCTS

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RESUME

De maintes coproduits à haute valeur ajoutée demeurent non valorisés. Cette étude a été proposée dans le but de valoriser les coproduits marins spécialement ceux issus des crustacés. Trois espèces de crustacés ont été étudiées, notamment, deux espèces de crevettes, *Parapenaeus longirostris* et *Penaeus kerathurus* mais également l'espèce de *Squilla mantis* a été également étudiée. Un essai à partir d'une source végétale a été effectué à partir des raquettes de figuier de barbarie *Opuncia officinalis*.

La concentration de l'enzyme et le fractionnement a été effectué en utilisant le sulfate d'ammonium à différentes saturations, une centrifugation à froid et à 17000rpm a été utilisée. L'activité enzymatique de la DAO dans l'extrait brut et dans les différentes étapes de fractionnement a été mesurée en utilisant la Putrescine comme substrat et mesurée par spectrophotomètre à une longueur d'onde de 340nm.

Des méthodes de chromatographies sur gel ont été utilisées pour la séparation des protéines en fonctions de leurs tailles (SEC, HPLC). La caractérisation de l'enzyme a été effectuée en utilisant la technique de SDS-PAGE et la technique d'électrophorèse On-chip. Le Spectre d'absorption à transformée de Fourrier a été déterminé.

ABSTRACT

Several coproducts with high added value are nowdays not valued. This study was proposed with the aim of enhancing marine coproduts especially from crustacean. Three species of crustacea, two species of shrimp (*Parapenaeus longirostris* and *Penaeus kerathurus*) as well as *Squilla mantis* were used in order to extract and purify the diamine oxidase enzyme (DAO). An essay was also done from a vegetarian source using prickly pear racket of *Opuncia officinalis* as specie.

Concentration and fractionation were performed by the addition of ammonium sulphate, $(NH_4)2SO_4$ saturation in stages and cold centrifuged at 17000 rpm. Enzyme activity of DAO crude and DAO fractionation results were tested using substrate Putrescine and measured with a spectrophotometer at a wavelength of *340 nm*.

Gel chromatographic methods were used for the separation of proteins and a Size Exclusion Chromatography (SEC, HPLC) method was used for the purification of the enzyme. The characterization of the enzyme was done by SDS-PAGE and on-chip Electrophoresis. The Fourier transform infrared spectroscopy (FTIR) spectrum was determined.

INTRODUCTION

Diamine oxidases (amine: oxygen reductase, deaminating copper containing EC 1.4.3.6) are enzymes eventually distributed among living organisms (Rinaldi & al, 1982). These enzymes have been often considered as histamine scavengers in animal tissues (Waton, 1956), hence the trivial name "histaminase" was attributed to them (Best, 1929) (Buffoni,1966). Alasalvar (2011) demonstrated the availability of the enzyme from marine coproducts.

Moreover the plant enzymes are very active toward diamines but less toward histamine.

This enzyme catalyzes the oxidative deamination of primary amines to form an aldehyde, ammonia and hydrogen peroxide as shown in reaction (Abdul & *al*, 2015).

$$R.CH_2.NH_2 + O_2 \text{ --- > } R.CHO + NH_3 + H_2O_2 \quad [1]$$

The reaction consists in the formation of a Schiffbase between the primary amino group and the carbonyl group of the enzyme followed by a proton subtraction from the adjacent a-carbon which is th elimiting step of the reaction (Buffoni & al, 1978) (Buffoni & al,1981)

DAO enzyme has been found in several microorganisms such as bacteria (Gale, 1942) (Satake & al, 1953), fungi, and the variety of plants (Liu & Liu, 2004) (Smith 1988) and animals (Choudhary,1999).

DAO enzyme is also widely found in plants such as beans, mung bean and pea sprouts (Wimmerova & Macholan, 1999). An essay from *Opuncia officilnalis* was envisaged in our study.

This study was conducted to isolate and characterize the DAO enzyme from different species of crustacean: *Parapenaeus longirostris, Penaeus kerathurus* and *Squilla mantis* moreover from vegeterian source: *Opuncia officinalis.*

In the present study we describe the steps of the purification of diamine oxidase.

MATERIALS AND METHODS

Materials

Materials used in this study are crustacean species *Parapenaeus longirostris, Penaeus kerathurus* and *Squilla mantis*and vegeterian source: *Opuncia officinalis.* Na-Phosphate buffer, Sephadex G-25 and G200, BSA (Bovine Serum Albumin), BRADFORD Solution, Putrescine, Histamine, GLDH, NADH, standard DAO.All other chemicals used were obtained as pure commercial products.

Isolation of DAO enzyme from crustacean coproducts

Crude extract of DAO enzyme

The coproducts were obtained from fresh crustacean without any pretreatment especially with bisulfites which are used as antioxidant to avoid the process of "black spot" which can be seen on crustacean samples. The three species were manipulated with the same procedure and were collected from the port of La Goulette. Then transported to laboratory, all manipulations were carried out at 0°C to avoid the degradation of the enzyme.

The cephalothorax and teguments obtained from *Parapenaeus longirostris, Penaeus kerathurus* and *Squilla mantis* were ground in cold condition by using a blender and one part of each homogenate was then added to 3 volume parts of Phosphate Buffered- Saline (PBS) [0.1M]. The homogenates were then incubated at 4°C during 3hours. After incubation a centrifugation step (17000 rpm, 4°C, and 30 minutes) is used to obtain the crude enzyme extract from each specie. The supernatant "crude extract of DAO" was then sored at -20°C.

Ammonium sulphate Fractionation: Differential precipitation

The DAO enzyme crude extract was fractionated with ammonium sulfate using successively 30% and 70% ammonium sulfate saturation. The goal of this step is to insure the separation of diamine oxidase present in our crude enzyme extract from other contaminants. Centrifugation was carried out using a SIGMA 3-30 KS centrifuge (17000rpm, 20 min, 4°C). The supernatant of the 30% precipitation was then collected to ensure the 70% precipitation. The final pellet containing diamine oxidase was then stored at -20°C.

Enzyme Assay

Diamine oxidase activity was tested with spectrophotometric assay like described on a SIGMA ALDRICH data Sheet for the standard of DAO using *BioTek Microplates Spectrophotometer*. The reaction was carried out at 37°C using a thermostatic incubator. The standard reaction mixture (1ml) contained deionized sterilized water, Na-Phosphate buffer [1M] pH=7.4, Glutamate Dehydrogénase GLDH (10mg/ml),α-Ketoglutarate acid 300mM, NADH (10mM) and the amount of sample added. The reaction was starting by adding substrate Putrescine (50nM), and then incubates during 30 minutes on incubator with DO reading at 340nm in 5 min intervals. A control sample using Diamine Oxidase from porcine kidney was done.

For each molecule of substrate converted by DAO one molecule of NADH oxidized in the coupled reaction. The change of absorbance per time ($\Delta E/\Delta t$) has to be determined in the linear range of the reaction. The specific activity (SA) of diamine oxidase can be calculated by dividing by the amount of protein present in the cuvette was expressed in units/mg. One unit (U) converts 1 µmol of substrate per minute.

Dosage of protein amount

The amount of soluble protein was determined using the BRADFORD method (Bradford, 1976) The lecture was carried out with *bioTekMicroplate Spectrophotometer.*

Gel Filtration Chromatography

Gel Filtration Chromatography was used to ensure the size separation of proteins and diamine oxidase present in the pellet collected from the first step. Gel filtration chromatography was carried out using a Sephadex G25 resin as stationary phase, a glass column*SaCo*(10ml), a peristaltic pump (*Gilson Minipuls Evolution*). The mobile phase used was Na-Phosphate Buffer. The flow rate used was 14ml/h. The fractions were collected using an automatic collector (Biorad 2110 model).

The Absorbance at 280 nm from the different fractions was determined.

HPLC Analysis

The fractions collected were passed on HPLC (1200 *Infinity Agilent System)* to ensure at first a size exclusion separation using *GMB BIOSpher*column. In a second time fractions were passed on C18 column to ensure separation. Data analysis, calibration and the analysis of the enzyme purity were done with the *chemstation software.*

Gel Filtration Chromatography

The fractions collected from HPLC analysis were passed on a second gel filtration chromatography and was carried out using a Sephadex G200 resin (Sigma Aldrich) as stationary phase. The other conditions were the same used on step2.

Characterization of the purified enzyme

SDS-PAGE

For the determination of the oligomers of the protein we insured a sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE). The stunning gel was 5% saturation and the running gel with 7% saturation. A *BIORAD System* was used.

Electrophoresis on-Chip

For the determination of the molecular weight and the purity of the enzyme, an On-ship electrophoresis was investigated using a *BioAnalyzer Agilent 2100 System*. The different fractions of each step were passed.

FTIR Analysis

The FTIR Analysis for the determination of the infrared spectra and so the purity of the enzyme was determined using Cary 630 FTIR Spectrometer.
The data analysis and the treatment of the spectra were done using *Agilent MicroLab Software*. The fractions collected after HPLC separations were analyzed.

Study of the purity with HPLC

The purity of the enzyme was determined using the *Agilent chemstation software*. The determination of the maximum UV-Visible absorption of the purified enzyme was determined.

RESULTS

Crude Extract

DAO enzyme extraction from different species in the crude extract is shown in Table I. The protein content of the crude extract of 399.44 mg and 0.15U/ mg of DAO enzyme activity in *Penaeus kerathurus,*292.630 mg and 0.25 U/ mg of DAO enzyme activity for *Parapenaeus longirostris,*177 mg and 0.035 U/ mg of DAO enzyme activity for *Squilla mantis* and 604mg and 0.083 U/mg of DAO enzyme activity for *Opuncia Officinalis.* These results are quite large and can proceed to the next stage of fractionation with ammonium sulphate.

As it is known that ammonium sulfate is a salt that is commonly used in the method of purification and concentration of enzyme. This is due to ammonium sulfate has several advantages such as high solubility, low-cost, low toxicity to most of the enzyme and has a stabilizing effect on some enzymes (Doris, 2000).
The addition of ammonium sulphate was done slowly at a temperature of 4°C while in-stirrer due to the increase in temperature due to the dissolution process assisted magnetic stirrer can cause denaturation and solubility changes.

Table I: The determination of protein concentration and specific activity (SA) in crude extract of different species.*TEA:Total Enzymatic activity, PF: Purification Factor*

Species	TEA **U/ml ***	Soluble protein **mg**	SA **U/mg**	Yield **%**	PF
Penaeus kerathurus	**60**	**399.44**	**0.15**	**100**	**1**
Parapenaeus longirostris	**73.15**	**292.63**	**0.25**	**100**	**1**
Squilla mantis	**6.3**	**177**	**0.035**	**100**	**1**
Opuncia officilnalis	**50.3**	**604**	**0.083**	**100**	**1**

Ammonium Sulfate Fractionation

Fractionation with ammonium sulfate saturation was made with 30% and 70 % of saturation. The results obtained are showed on the table below (table II). In the process of fractionation, the *Parapenaeus longirostris* sample showed the highest specific activity with 0.32 U/mg and a Yield of 54.73% followed by *Penaeus kerathurus* with 0.167 U/ml specific activity.
The Yields (%) obtained, which are around 60 % , acceptable but not too high may be due to the presence of impurities in the mixture.

Table II: The determination of the concentration of protein and the specific activity in the different species after ammonium sulfate fractionation. *TEA:Total Enzymatic activity, PF: Purification Factor*

Species	TEA **U/ml ***	Soluble protein **mg**	SA **U/mg**	Yield **%**	PF
Penaeus kerathurus	**25**	**149.32**	**0.167**	**41.66**	**1.1**
Parapenaeus longirostris	**41.7**	**130.3**	**0.32**	**56.73**	**1.3**
Squilla mantis	**4.2**	**86**	**0.048**	**66.66**	**1.4**
Opuncia officilnalis	**30.7**	**163**	**0.188**	**61.3**	**2.3**

An example of the enzyme kinetic for *Parapenaeus longirostris* obtained is shown on the figure 1. The determination of the specific activity for all species is done on the linear zone of the curve.

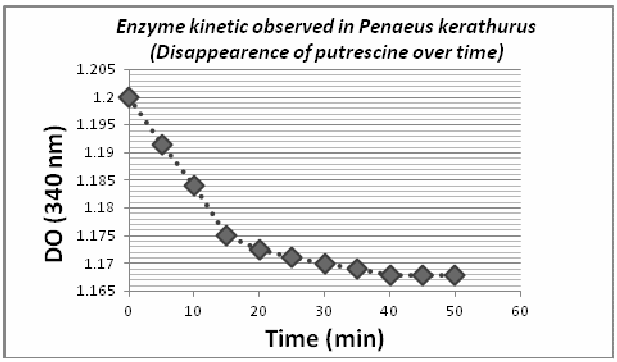

Figure 1 : The enzymatic chenetic (disparition of putriescine) determined on the crude extract of *Penaeus kerathurus.*

Gel filtration Chromatography

The results of the protein content of each fraction measurements are determined. Gel filtration is intended to separate the enzyme proteins by size (Holstein, 1975).Molecules move through porous granular layer, smaller molecules spread farther into the pores and therefore move more slowly, while larger molecules move faster.

To facilitate reading and see the tendency of each fraction of the protein content using UV-Visible absorption. The curve measuring the protein content of 70% saturated (fractions 1-14) can be seen in Figure 1. This step was done for all other species.

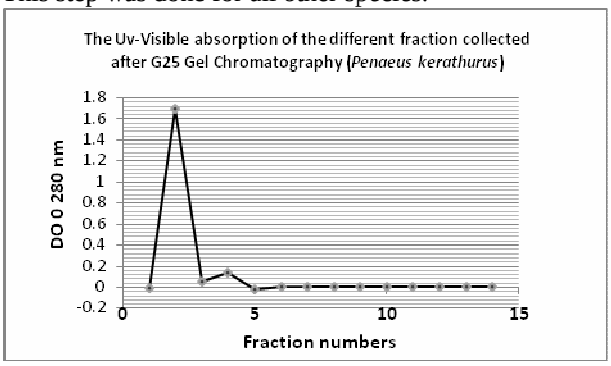

Figure 1: UV-Visible absorption of the different fraction collected after G25 Chromatography (*Penaeus kerathurus)*

Figure 1 shows that there was a peak (high protein) in fraction number 3 with 1.7DO on 280 nm. This step was done for all other species and the amount of proteins and the specific activity were then determined. The specific activities of the highest pics were calculated fo each especies and the results are shown on table III.

Table III: The determination of the concentration of protein and the specific activity in the different species after G25 Gel Filtration Chromatography. *TEA:Total Enzymatic activity, PF: Purification Factor*

Species	TEA **U/ml ***	Soluble protein **mg**	SA **U/mg**	Yield **%**	PF
Penaeus kerathurus	**10**	**17.5**	**0.571**	**36.66**	**3.8**
Parapenaeus longirostris	**30.16**	**20.8**	**1.15**	**41.03**	**5.8**
Squilla mantis	**3.1**	**32**	**0.096**	**49.2**	**2.7**
Opuncia officilnalis	**20.6**	**104.82**	**0.196**	**40.95**	**2.4**

Table III shows that the protein content is not directly proportional to the DAO enzyme activity. The highest specific activity was found for *Parapenaeus longirostris* with 1,15 U/mg followed by 0,571 U/mg for *Penaeus kerathurus*, 0,192 for *Opuncia officinalis* and a the lowest level was observed for the *Squilla mantis* with 0.096 U/mg. The raising of the specific activity of DAO occurs because the DAO enzyme protein has experienced separation from the other components of the protein, thus also has higher specific activity.

HPLC Analysis

Size exclusion chromatography : SEC-HPLC Analysis

The determination of the purity of the fractions collected from the G25 Chromatography was done by HPLC using the affinity principle with a specific column. The chromatograms obtained are shown on figure 2.

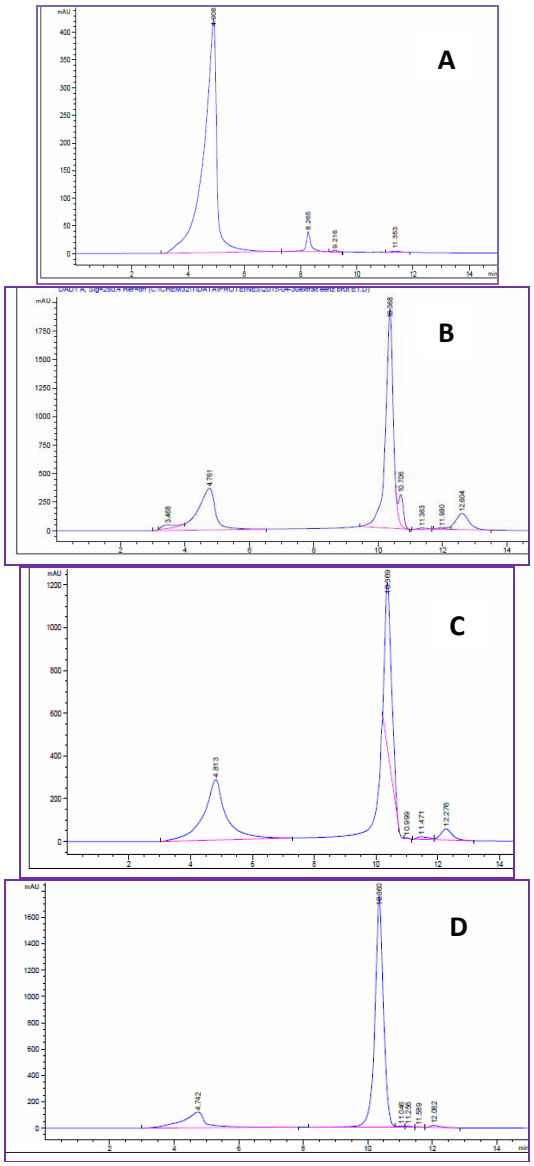

Figure 2 : SEC-HPLC Analysis, Chromatogram of DAO Standard (**A**), chromatogram of *Penaeus keraturus* Fraction analysis (B)chromatogram of *Parapenaeus longirostris* fraction analysis (**C)** chromatogram of *Squilla mantis* fraction analysis (**D**).

The concentrations obtained were verified after establishing the calibration curve of the enzyme, chromatograms are in correlation with the results shown on table 3.
The results obtained are in correlation with the specific activity calculated for each species. The high of the peak (4.908 min) corresponding of DAO, is directly correlated with the concentration of the enzyme.

Cl8-HPLC Analysis

All the fractions were passed on C18 column for the separation considering the hydrophobicity with the stationary phase. Figure 3 shows an example of chromatogram obtained after G25 gel Filtration chromatography for *Penaeus Kerathurus*.

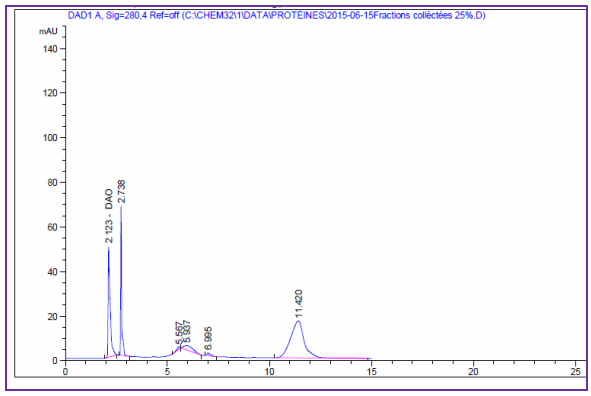

Figure 3: C18 HPLC separation for G 25 gel filtration chromatography fraction of *Penaeus keraturus*. DAO was eluted on 2.123 minutes.

This step was repeated a lot of times for the concentration and the collection of the enzyme using an automatic collector. The Vials containing the purified enzyme were then stored at -20°C.

Characterization of the purified enzyme

Determination of DAO UV-Visible absorption

A spectra analysis was done with HPLC and the maximum of the absorption was determined. Figure 4 show the 3Dimentionnal analysis and DAO has a maximum absorption of 480nm in the visible domain and 278 nm in the UV domain.

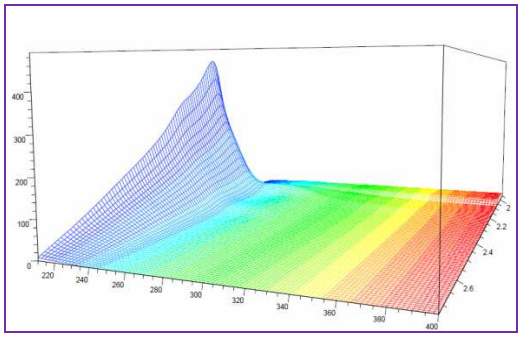

Figure 4: the 3 Dimensional analysis of the UV-Visible absorption of Diamine Oxidase.

FTIR Analysis

The infrared spectra of the standard and the purified enzyme were determined. Figure 5 shows a high degree of similitude between the standard used the purified enzyme, with essentially the identification of amides groups, a major responsible of the peptides bond.

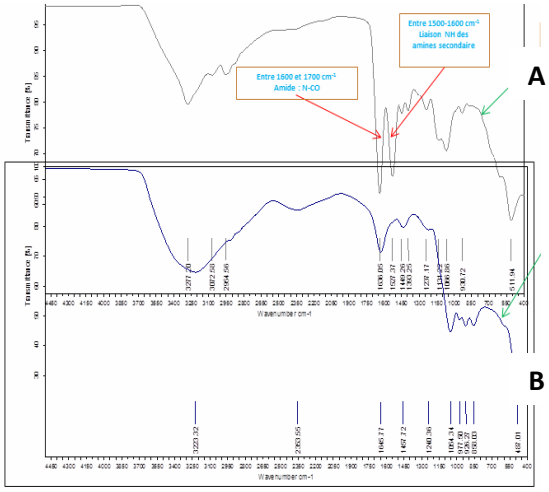

Figure 5: the infrared spectra of the DAO standard (A) and the purified enzyme (B).

On-chip Electrophoresis

The on chip-electrophoresis of the DAO standard with a same principal of SDS-PAGE like described (Laemmeli, 1970) reveals the presence of two equal bonds as the arrows on the figure shows, which can correspond of the dimers of the enzyme. The two subunits have a size of 30K.Da. Figure 6 shows the electropherograms obtained.

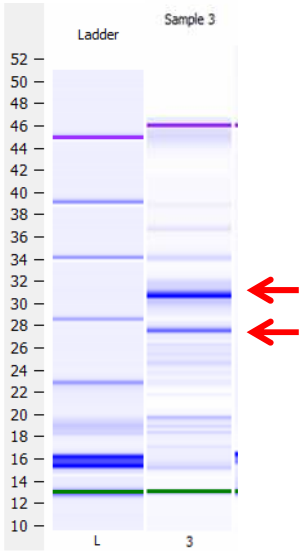

Figure 6 : The electropherogramm of the purified Diamine oxidase(Sample 3) and the ladder used (L)

DISCUSSION

In the present study we described a new purification scheme using firstly a classic purification method (ammonium sulfate precipitation, gel chromatographic separation) but also we have used techniques with high performance (HPLC) to insure the purification and to study the purity of our enzyme, these techniques (SEC-HPLC and C18-HPLC) exploits the strong affinity of the enzyme with the type of column used and allows a rapid purification.
Due to the relatively high expression level of DAO in seafood co-products (Ienistea, 1973) we choose these tissues as starting material to develop a purification scheme for this protein. The new procedures especially on the characterization methods used for the enzyme takes advantages of several proprieties of DAO concerning protein stability, specific affinity and conservation of enzymatic activity.

A lot of studies describing the purification of this enzyme were done but are difficult to compare, because of the deference of the DAO assays used and less sensitive assay methods to determine the enzymatic activity but also no evidence was provided for the homogeneity of the products (Doris,2000), (Kluetz, 1977), (Rinaldi & al, 1982).

Several chromatographic methods were also used for the purification of this enzyme (Munis & al,1998) but procedure was used for crude material and gave a low yield.

In summary, the combination of tow affinity chromatography allowed a scheme simpler and faster than the use of other steps such as the anion exchange chromatography as described on (Doris, 2000). The availability of sufficient pure protein, the use Ultrapure water as mobile phase, despite the number of repetitions necessary to concentrate the enzyme, will facilitate future studies to elucidate the structure of this enzyme and its functions in various metabolic pathways.

CONCLUSION

The steps of purification applied on different species are specific and lead to an acceptable purification. *Parapenaeus longirostris* shows the highest specific activity with 1.15U/mg followed by *Penaeus kerathurus* with 0.571 U/mg and 0.196 U/mg for *Opuncia Officinalis.*
For the reached level we need to characterize the DAO purified and to determine the biochemical characteristics and its catalytic constants (*Km,Vm*).

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