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Competitive Spectrophotometry for Microbial Dipeptide Transport Systems

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Portage kinetic constants of peptide transport can be measured by competitive spectrophotometry. The kinetic constants of L-Glu-L-Glu transport in *Escherichia coli* were ascertained using L-Phe-L-3-thia-Phe (PSP) as a detector. Since the production of thiophenol upon intracellular hydrolysis of PSP was competitively inhibited by L-Glu-L-Glu, it was able to compute the kinetic constants of L-Glu-L-Glu using this method. The resulted data were in agreement with the values obtained by the method of Michaelis-Menten kinetics. The potential of this method was examined against dipeptide transport systems in various microorganisms. These results strongly suggest that the overall properties of individual systems for dipeptide transports can be easily characterized by competitive spectrophotometry.

There are many examples of peptides in nature, containing molecules (in some cases, as their side chains) that are normally impermeant but are biologically active (7, 26, 27). This indicates that there must be relevant systems tolerable against these unusual peptides. Impermeable molecules are either attached to peptide backbones, or substituted to peptide α-glycine moieties. After transport, these molecules are released upon hydrolysis by intracellular peptidases (16).

Peptide transport systems are widely distributed in microorganisms (20), and their abilities to recognize these unique structures are varied. Transport systems for di- and oligopeptides (include tripeptides) (18) have been extensively studied in *E. coli*. The oligopeptide transport system in this organism is regarded to have relatively low specificity against peptide side chains, suggesting that the intracellular permeability of impermeable compounds can be readily increased if they are delivered as components of oligopeptides (2, 13, 19). On the contrary, their dipeptidyl deliveries are not recommended because the correspondent permeases have been thought to be stringent against peptide side chains (13). In our previous report (11), however, it was shown that normally impermeable molecules could be transported effectively even through microbial dipeptide transport systems by increasing their hydrophobicities. Moreover, their transports were exclusively contingent on the peptide compositions. These observations indicate microbial dipeptide transport systems presumably contain unique specificities against substrate transports. In this regard, it is strongly suggested that the overall studies on the kinetic properties of individual transport systems are prerequisite for the purpose of constructing an appropriate peptide carrying the target molecule. To do this, one would anticipate an easy way to assay the peptide transport.

The peptide in which an internal or C-terminal glycine residue bears a nucleophilic α-substituent was first developed by Kingsbury *et al.* (16). Intracellular hydrolytic release of the glycine amino group allows the leaving group to be expelled. In devising detector peptides, the use of chromogenic nucleophiles was proposed for enzyme assay (14). As peptide mimicries, chromogenic peptides have potentialities assessing biological reactions. Perry, *et al.* reported the use of di- or tripeptides attached by sulfhydryl compounds to determine transport affinities for peptides in *E. coli* (24). However, since the factor affecting their transports is not only limited in Km, but also includes Vmax, it is anticipating if the Vmax can be evaluated by using detector peptide.

The competitive spectrophotometry was first introduced to determine both kinetic constants of a given substrate using detector in a single experiment (10). This method proved to be particularly useful in ascertaining kinetic powers of various substrates under the same experimental conditions. Using this method, a promising result was recently obtained from the portage transport of peptides in *E. coli* (8). In this study, we examined the

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feasibility of the competitive spectrophotometry to investigate the microbial portage kinetics for dipeptide transports, and extended this to characterize overall properties of microbial dipeptide transport systems.

MATERIALS AND METHODS

Microorganisms and Culture Conditions

Escherichia coli No.20 (15) and *Staphylococcus aureus* No.19 (5) were stock strains in our laboratory. Strains of *Pseudomonas aeruginosa* 1630 and *Candida albicans* 1940 were obtained from KCTC, Korea. Cell growth was carried out in a nutrient medium containing 0.5% polypeptone and 0.3% yeast extract, pH 7.0, to late logarithmic growth phase (O.D. at 660 nm=0.8~1.2) using a water bath shaker at 30°C, 90 rpm.

Intact Cell System

Following procedures were carried out at 0~4°C. Cells were harvested by centrifugation at 10,000 g for 10 min, and washed twice by centrifugation with 50 mM phosphate buffer (PB), pH 7.0. Intact cell systems were prepared by resuspending in 50 mM PB (pH 7.0) to give a standard turbidity of 1.0 at 660 nm, and stored on ice before use. Cell suspensions perpared this way showed no significant changes in cell densities after incubation for several hours at 30°C (17).

Single Substrate Kinetics

Intact cell systems were dispensed into test tubes on ice, and 0.1 ml of L-Glu-L-Glu solution (0.152~0.608 mM) was added to each to give a final volume of 1ml. Intracellular uptake was initiated by transferring test tubes onto water bath at 30°C. Aliquots of the samples were taken before incubation. After 10 min of incubation, the transport of L-Glu-L-Glu was stopped by placing the tubes on ice. Cell suspensions were then centrifuged at 0°C (10,000 g, 2 min) to remove cell debris. The resulted supernatants were subjected to DEAE-cellulose column chromatography (0.6×3.5 cm; 5 mM PB, pH 7.0), washed, and fractionated by increasing the concentration of NaCl. Elution profiles were achieved by measuring the amount of primary amino groups at 420 nm using 2,4,6-trinitrobenzenesulfonate (TNBS; ε_{420}=10,000/M/cm) (3) as described previously (9). Kinetic constants for L-Glu-L-Glu and L-Glu were determined using the Lineweaver-Burk plot.

Competitive Spectrophotometry of Peptide Transport

Typically, 1 ml of the intact cell system containing 0.1 mM L-phenylalanyl-L-3-thia-phenylalanine (PSP) and 0.1 mM 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) (6) was incubated at 30°C with or without L-Glu-L-Glu at appropriate concentration, followed by time course production of 3-carboxy-4-nitro-thiophenolate (ε_{412}=13,600/M/cm) (14) at 5-min intervals. Due to the presence of L-Glu-L-Glu, additional incubation time was required to achieve the same extent of thiophenol production observed by PSP alone. And, it was able to measure the time lag (Δt) between two progress curves during the steady state of transport. Accordingly, the kinetic constants for this competing peptide could be evaluated as described previously (8).

Chemicals

PSP (14) was prepared by replacement of acetoxy group in Cbz-L-phenylalanyl-2-acetoxyglycylbenzyl ester with thiophenol, followed by deprotection with HBr in glacial AcOH. H^1 NMR (D_2O-DMSO) δ 2.8-3.1 (d, 2H), 3.9 (t, 1H), 5.3 (S, 1H), 7.2-7.4 (m, 10H). L-Phenylalanyl-L-2-sulfanilylglycine (PSG) was prepared as described previously (12). H^1 NMR (D_2O-DMSO) δ 2.9-3.0 (d, 2H), 4.0-4.1 (t, 1H), 5.5 (s, 1H), 6.5-7.5 (m, 9H). TNBS, DTNB and dipeptides were purchased from Sigma Chem. Co., USA. Thiophenol was obtained from Junsei Chem. Co., Japan. All other reagents were commercial preparations of analytical grade.

RESULTS

Determination of Dipeptide Transport by Michaelis Kinetics

Microbial uptake of primary amine compounds such as amino acids was shown to be determined by using their intact cell systems, and those initial uptake rates were changed by cell density (17). An experiment to optimize the intact cell density was carried out using PSG. As can be seen in Table 1, optimal cell densities for PSG uptake varied with individual organisms. Therefore, we used a cell system with turbidity of 1.0 at 660 nm for standardization. PSG could not be used for the purpose of ascertaining transport kinetics of the dipeptide transport system in *E. coli*, because the production of sulfanilic acid after the addition of PSG perturbed to determine PSG in extracellular medium. Similar phenomena were also noted when other dipeptides were used. For this reason, L-Glu-L-Glu was selected as a testing substrate for the analysis of transport kinetics in *E. coli*. Because of its characteristic charge, this peptide could be discriminated from its intracellular product, L-Glu, by using DEAE-cellulose column chromatography under low

Table 1. Effect of cell density on the PSG uptake.

Cell density (O.D. at 660 nm)	Uptake rate (nmloes/min)			
	E. coli	*S. aureus*	*P. aeruginosa*	*C. albicans*
0.5	0.19	0.46	N.D	0.32
1	0.46	0.50	0.46	0.61
2	0.58	0.93	0.19	0.19
3	0.19	0.16	0.23	0.20

N.D: not determined. For details, see Materials and Methods.

ionic strength. Fig. 1 illustrates the decrease in the extracellular L-Glu-L-Glu was accompanied by the increase in the extracellular L-Glu. Individual fractions were separately combined, and total amounts before and after incubation were measured. Under the constant cell density, the relative transport rates of these compounds were increased by increasing the concentration of L-Glu-L-Glu, but not proportionally (Table 2). A plot according to the Lineweaver-Burk method gave a straight line for the L-Glu-L-Glu transport, resulting a Km of 0.83 mM and a Vmax of 3.08 nmoles/min (Fig. 2). Kinetic constants for L-Glu secretion were also evaluated, but their values were different from the above values as 0.7 mM and 2.94 nmoles/min of Km and Vmax values, respectively. This finding suggests that there must be a controlling system for L-Glu secretion.

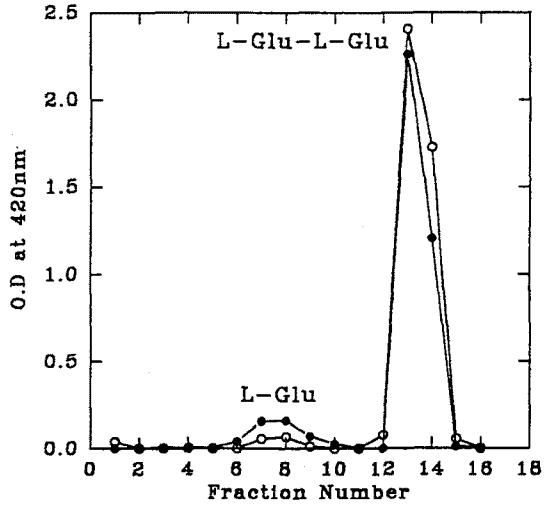

Fig. 1. Determination of L-Glu-L-Glu transport by DEAE-cellulose chromatography.

0.456 mM L-Glu-L-Glu was used for transport in intact cell system of *E. coli*. Incubation was carried out at 30°C for 0 min (○) or 10 min (●). Cells were removed by contrifugation (10,000 g, 10 min), and the supernatants were applied to a column of DEAE-cellulose chromatography (0.6 cm×4 cm). Elution profile was observed by measuring the content of primary amino group using TNBS as described in Material and Methods.

Table 2. Effect of L-Glu-L-Glu concentration on the transport of L-Glu-L-Glu in *E. coli*.

L-Glu-L-Glu (mM)	Transport rate (nmoles/min)	
	L-Glu-LGlu	L-Glu
0.152	0.517	0.508
0.304	0.638	0.890
0.456	1.033	1.218
0.608	1.323	1.388

Experimental conditions were in Fig. 1.

Determination of Dipeptide Transport by Competitive Spectrophotometry

The transport of L-Glu-L-Glu in *E. coli* was determined by using the competitive spectrophotometry (10). To achieve relevant distances (Δt) between two progress curves (see Materials and Methods for details), L-Glu-L-Glu was added near 10 times than PSP (Fig. 3). The rate of thiophenol release by the intact cell system from PSP was monitored spectrophotometrically by measuring its reaction with DTNB as described in Materials and Methods. As indicated in Fig. 3, the extracellular liberation of thiophenol showed no discernible lag after the PSP transport was initiated (see slopes at the begining of incubation). The thiophenol is thought to be diffused rapidly across the cell membrane as a small lipophilic molecule (24). In other words, the rate of production of thiophenol corresponds to the rate of PSP transport. To ascertain this notion, the kinetic constants for PSP transport were separately determined, employing single substrate kinetics. And the data were compared with the result from the DTNB method. Both data were identical with a Km value of 0.022 mM and a Vmax value of 3.3 nmoles/min. Accordingly, the additional time (Δt), occasioned by the presence of L-Glu-L-Glu, was plotted as a function of $[(So-y)/So]^n$. n is the relative ratio of kinetic power of L-Glu-L-Glu against PSP, and So, y are concentrations of the detector and product, respectively. The kinetic constants were computed by accessing true n value. A straight line was obtained when n value was

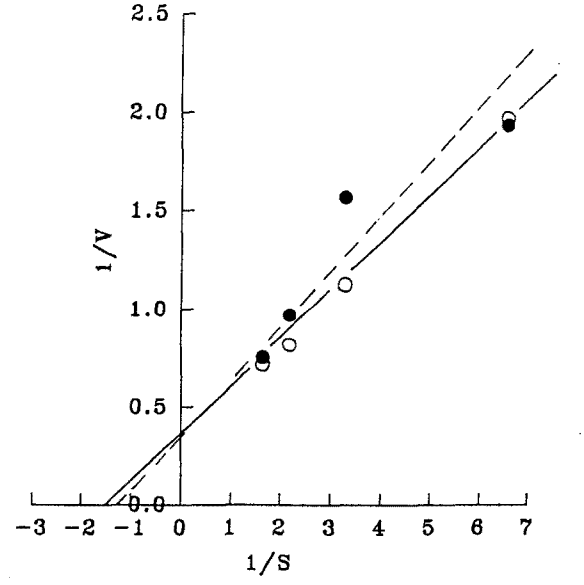

Fig. 2. Determination of kinetic constants of L-Glu-L-Glu transport and L-Glu-L-Glu secretion in *E. coli*.

Lineweaver-Burk plot was made using the data in Table 2. (○-○), L-Glu-L-Glu; (●-●), L-Glu.

0.025 (Fig. 4). With known kinetic constants of PSP, a Km of 0.81 mM and a Vmax of 3.04 nmoles/min were evaluated for the transport of L-Glu-L-Glu. These values are in good agreement with values obtained by the single substrate kinetics as described in above.

Extension of the Use of Competitive Spectrophotometry

The potential of competitive spectrophotometry was examined by applying to characterize the overall properties of microbial transport systems against various dipeptides. Kinetic constants for dipeptide transports in various microorganisms were determined, and the data are presented in Table 3. Previously, the N-terminal composition of dipeptides was shown to be concerned with dipeptide transports (11). In this study, however, microbial dipeptide transport systems appeared to have specificities against C-termini also, although individual systems varied in ability to recognize the moieties (Table 3). For instance, the kinetic power of L-Leu-X was increased mostly by increasing the hydrophobicity of X moiety. In either strain of *S. aureus* or *P. aeruginosa*, the increase in the kinetic powers was over 20 times by this substitution. Nevertheless, their ways to increase kinetic powers were distinguishable and different in their potential to change Km and Vmax values for a given substrate. Interestingly, the dipeptide transport system of *S. aureus* could not distinguish PSP from L-Phe-L-Phe for transport among those tested. It is noteworthy that the dipeptide transport system of *P. aeruginosa* in general binds dipeptides with much greater affinities than other

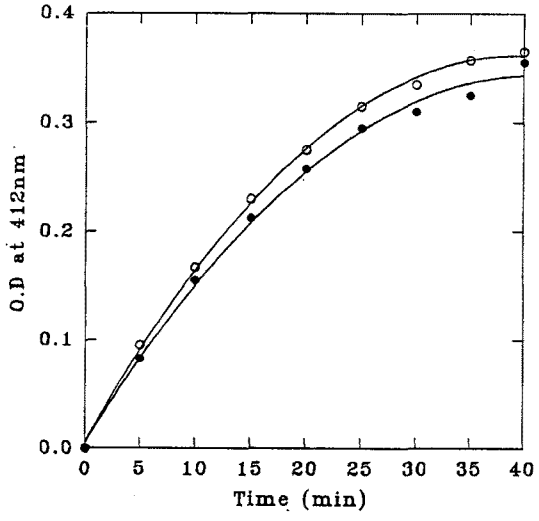

Fig. 3. Transport competition between PSP and L-Glu-L-Glu in *E. coli*.

For the upper curve (○), the intact cells suspended in 50 mM PB (pH 7.0) consisted of 10 μl of 10 mM DTNB and 10 μl of 10 mM PSP in a final volume of 1 ml (A_{660}=1.0). For the lower curve (●), the conditions were identical but in addition 100 μl of 10 mM L-Glu-L-Glu was present. In both instances the reactions were initiated by the addition of PSP.

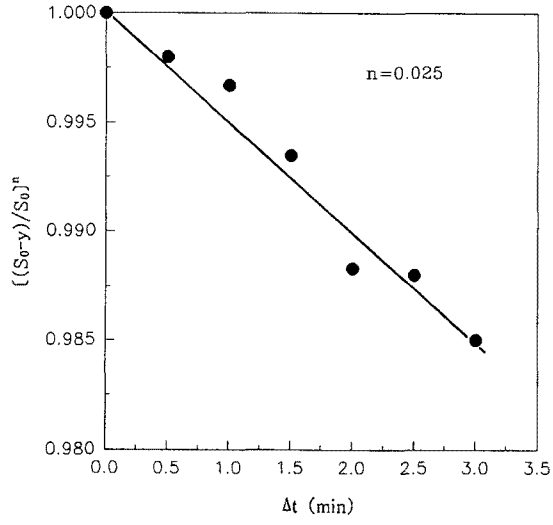

Fig. 4. Evaluation of the ratio of kinetic powers of L-Glu-L-Glu and PSP.

The additional time required to achieve the same extent of hydrolysis of PSP due to the presence of L-Glu-L-Glu (see Fig. 3) was plotted as a function of $[(S_o-y)/S_o]^n$.

Table 3. Determination of kinetic constants for dipeptide transports in various microorganisms by competitive spectrophotometry.

Compound	*E. coli*		*S. aureus*		*P. aeruginosa*		*C. albicans*	
	Vmax	Km	Vmax	Km	Vmax	Km	Vmax	Km
PSP	3.3	0.022	3.4	0.070	0.98	0.038	2.2	0.032
Phe-Phe	4.8	0.032	3.5	0.071	0.90	0.017	2.8	0.081
Phe-Ala	6.2	0.042	3.3	0.110	1.80	0.017	1.1	0.016
Phe-Leu	5.7	0.038	2.9	0.049	0.38	0.015	4.0	0.058
Ala-Phe	6.8	0.015	2.8	0.047	0.38	0.015	1.7	0.024
Leu-Phe	6.0	0.013	3.5	0.035	0.26	0.011	2.2	0.032
Leu-Pro	3.0	0.134	3.0	0.040	0.11	0.014	1.2	0.067
Leu-Leu	2.7	0.018	2.4	0.032	1.50	0.008	0.9	0.043
Leu-Asp	3.6	0.048	1.9	0.380	0.20	0.025	0.9	0.137

For conditions, see Materials and Methods.

systems, accompanied in most cases by decrease in Vmax. Also, this strain was the only exception to give poorer affinity against PSP than L-Phe-L-Phe. Mention should also be made about the resemblance of properties for peptide transports between prokaryotic and eukaryotic dipeptide transport systems. The substrate specificity for dipeptides shown in *C. albicans* was remarkably similar to those found in bacteria.

DISCUSSION

In our efforts aimed at delivering normally impermeable molecules by microbial peptide transport systems, we recognized the importance of portage kinetics in order to design peptide prodrugs. Unfortunately, microbial peptide transports in particular are difficult to investigate in following aspects: Unlikely to the transport of amino acids (1), microbial transports of peptides are usually accompanied with immediate production of their constituents upon intracellular hydrolysis by cytoplasmic peptidases (29), perturbing peptide assay in the extracellular medium. Moreover, the intracellular metabolism of liberated amino acids (include those labeled radioactively) affects the observed kinetics (21, 25). To circumvent these problems, an assay with fluorescamine was proposed to determine kinetic constants for peptide transport. But, it requires specialized apparatus (22). Therefore, it seems virtually impossible to determine accurately the portage kinetics of peptide transports with conventional methods. Also, in considering the overall delineation of the substrate specificities of a certain peptide transport system, one would anticipate a rather simple and rapid method of determining the peptide transport.

While seeking an appropriate way to evaluate Km and Vmax for peptide transports, it was recently shown that the competitive spectrophotometry would be useful for this purpose (8). In this study, we compared this method with single substrate kinetics, and indeed it proved to be useful for transport assay. The methodology implicated the technique, based on the detection, by DTNB, of extracellular thiophenol for PSP transport, was found to be extremely valuable in determining peptide transport. Because of its lipophilic nature, the production of thiophenol caused no discernible time lag after PSP was added (Fig. 3). Accordingly, it would not affect the observed kinetic constants. In fact, it is strongly recommended to obtain precise slopes in progress curves at the begining of the transport since this period is only under the steady state for the portage kinetics. This notion was elucidated in Fig. 4, where a straight line was achieved within 3 min of Δt. This observation suggests that under experimental conditions the virtual assessment of PSP transport can be accomplished within 10 min of incubation. A number of other chromogens were reported for α-glycine substituents in place of thiophenol, i.e., nitrophenol (16), (hydroxy) alkyl mercaptans (4) or sulfanilic acid (11). But, none of these were practically useful because of instability or difficulty in secretion.

The potential of this method was ascertained by measuring kinetic constants for dipeptide transports in various microorganisms (Table 3). Following our investigation we found some unique properties of microbial transport systems against dipeptides as follows: a) the existence of specificities against C-termini, b) relatively high affinities but low in Vmax with *P. aeruginosa*, c) resemblance in transport characteristics between bacteria and *C. albicans*, and d) differences in ability to recognize the mimicry of a peptide. The methodology shown here can be generalized toward any kinds of kinetic investigations in living systems by preparing pairs of compounds, where a substrate can be monitored without interruption by another substrate or its components.

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